AD_____

CONTRACT NUMBER DAMD17-95-C-5003

TITLE: Fermentation, Recover, and Purification of the Hc Fragment of the Botulinum Neurotoxin from *Pichia pastoris*

PRINCIPAL INVESTIGATOR: Michael M. Meagher, Ph.D.

CONTRACTING ORGANIZATION: University of Nebraska-Lincoln
Lincoln, Nebraska 68588-0430

REPORT DATE: March 1997

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 1997	Annual (15 Apr 96 - 14 Apr 97)

4. TITLE AND SUBTITLE
Fermentation, Recover, and Purification of the HC Fragment of the Botulinum Neurotoxin from Pichia pastoris

5. FUNDING NUMBERS
DAMD17-95-C-5003

6. AUTHOR(S)
Michael M. Meagher, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Nebraska-Lincoln
Lincoln, Nebraska 68588-0430

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This mid-term report is an update on the purification of *Clostridium botulinum* serotype B H_C fragment expressed intracellularly in *Pichia pastoris*, clone BBC-C-02136 A5 [BoNT B (Hc)/pHILD4]. During the first half of this year the fermentation was improved by the addition of casamino acids during the methanol feed. In addition, a process using a Streamline column (fluidized ion exchange column) was used to capture the H_c fragment directly from the cell homogenate at approximately 70 to 80% pure. This step replaces the polyethyelenimine precipitation and the ammonium sulfate precipitation step in the previous purification process. A purification process was completed that generates a 99+% pure product by SDS-PAGE and 97% to 99% pure on isoelectric focusing.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Botulinum, Hc Fragment, Vaccine, Pichia pastoris, Fermentation, Purification	51
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

(✓) Where copyrighted material is quoted, permission has been obtained to use such material.

(✓) Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

(✓) Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

(✓) In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

(✓) For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 32 CFR 219 and 45 CFR 46.

(✓) In conducting research utilizing recombinant DNA technology,the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Principal Investigator's Signature

Date 7/2/96

TABLE OF CONTENTS

INTRODUCTION

This mid-term report is an update on the purification of *Clostridium botulinum* serotype B H_C fragment expressed intracellularly in *Pichia pastoris*, clone BBC-C-02136 A5 [BoNT B (Hc)/pHILD4]. During the second quarter and part of the third quarter we evaluated a fluidized ion exchange column (STREAMLINE column from Pharmacia) as a replacement for the polyethyleneimine precipitation step and the ammonium sulfate precipitation step. Hydrophobic interaction chromatography (HIC) and anion exchange chromatography were evaluated for purification of a 47 kd contaminating fragment from the native BotNB Hc fragment. The fermentation was changed slightly by adding casamino acids during the methanol feeding which increased yield.

During the first part of the third quarter (December 11, 1996) a purification process was delivered to Walter Reed so that standard operating procedures can be developed for GMP production in April, 1997. The primary reason for the delay of this quarterly report was the effort put forth to deliver a scaleable process to Walter Reed. The process was not completely identified until the end of November, 1996. We believe at this point that we have a simple process that produces a product that is greater than 99% pure based on SDS-PAGE at an 8 μg load stained by coomassie blue. This result is confirmed by isoelectric focusing from pH 5 to 8.

FERMENTATION

Materials and Methods

A vial containing frozen culture is transferred into a test tube containing 10 mL of MGY and incubated at 30°C in a rotary shaker at 100 rpm. After 24 h the test-tube culture is used to inoculate shake flask containing 500 mL of medium consisting of 13.4 g/L yeast nitrogen base without amino acids, 20 g/L glycerol, 0.0004 g/L biotin and 0.1 M phosphate buffer of pH 6.0. The shakeflask is incubated at 30°C in the rotary shaker for approximately 25 to 30 h to a final OD_{600} = 20 before inoculating a fermenter with 2.5 L of initial volume.

The fermentation is performed in a basal salt media as described previously [1] in a 5 L BioFlo III fermenter (New Brunswick Scientific, Edison, NJ). At the end of the three hour fed-batch glycerol phase, 1 % casamino acids are added based on the initial fermentor volume. Also, at this stage the temperature set point is reduced to 25° C. The methanol feeding process is presented in Table 1. After 20 h of methanol feeding (OD_{600}=220) the cells are harvested by either centrifugation or cross flow membrane filtration and frozen.

Table 1. Methanol Feeding Process

Induction time (h)	Methanol feed rate (mL/L/h)
0	5
3	7
4	8
8	10
16	11
20	11

Induction samples were analyzed for Hc fragment by placing 100 mg of wet cell mass into a 2.0 mL eppendorf-type screw cap tube with 1 mL of breaking buffer (50 mM NaH_2PO_4, 1 mM EDTA, 5 % glycerol, 1 % PMSF, pH 6.0). An equal volume of zirconium-silica beads were added to the cell-breaking buffer suspension. The cells were disrupted in a Mini Beadbeater (Biospec Products, Bartlesville, OK) in 8 one-minute cycles in a cold room (4°C). The disrupted cell mass was centrifuged, the supernatant was treated with PEI, and the PEI supernatant was ammonium sulfate precipitated as described previously [1]. The protein precipitate from the ammonium sulfate treatment was redissolved in breaking buffer, the appropriate volume of SDS-PAGE sample buffer was added, and loaded (70 μg in 25 μL) onto an SDS-PAGE gel. Western Blot was performed according to Protocol 2 [1], with purified anti-BoNT B horse's anti-serum as a primary antibody (dilution 1:700), and anti-horse IgG as a secondary antibody (Kirkegaard and Perry, Gaithersburg, MA, 1:1500 dilution).

Results and Discussion

The profile of cell density and methanol feed rate vs time is shown in Figure 1 and indicates a steady continuous growth during the induction phase. A western blot of the time course during methanol induction is shown in Figure 2. The H_c fragment intensity continues to increase during the entire methanol induction, even after 20 h of methanol induction. The only difference from previous fermentations, where maximum

H_c fragment was produced after 12 to 14 of induction, was the addition of casamino acids just prior to the methanol feeding [1]. The addition of amino acids is recommended as a way to reduce the effect of proteases on the product. Another hypothesis is that there may be an amino acid deficiency that may have caused the degradation of the H_c fragment which was eliminated by the addition of casamino acids. In either case, the addition of casamino acids had a profound effect on the yield.

HOMOGENIZATION

Materials and Methods

One hundred grams of wet frozen cells were suspended in 1 L of cold lysis buffer (20 mM MES, 5 mM EDTA and 2 mM PMSF (pH 5.7)) and disrupted by passing through a APV Gaulin 30CD homogenizer at 14,000 psig. Preliminary studies were performed with a Y-110 Microfluidizer operating at 19,000 psig. The temperature of the homogenate for either unit prior to homogenization did not exceed 4°C. Studies with the Microfluidizer were performed as a demonstration at the request of UN-L.

Results and Discussion

Ten passes with the APV Gaulin 30CD resulted in a protein concentration of approximately 4 mg/mL. Based on visual inspection with a microscope, 4 mg/ml represented 50 to 60% disruption. Cells disrupted using glass beads on the small-scale produced 8 mg/ml. The microfluidizer was able to disrupt at nearly 95% efficiency, based on microscopic examination, and generated a soluble protein concentration of approximately 8 mg/ml after 6 passes. Based on these preliminary results the microfluidizer was more efficient than the Gaulin homogenizer. The yield of the purification processes would increase by 55 to 60% if 95 % disruption was achieved. It is recommended that a microfluidizer be further investigated based on our preliminary results.

STREAMLINE COLUMN

Material and Methods

The Streamline column is a single pass expanded bed adsorption operation in which proteins are recovered from crude feed stock or cell homogenate without the need for prior clarification. The column, which is 2.6 x 100 cm and contains 73 ml of SP Streamline media resulting in a packed bed height of 15 cm, is designed to run in two modes. In the first mode the specifically designed ion exchange resin is fluidized by flowing buffer at 300 cm/h in the upward direction producing a stable, uniformly expanded bed. The homogenate and buffers are cooled to 4°C, but the column is at room temperature, but is cooled by the buffers. The cell homogenate, which is at the same pH and the conductivity as the equilibration buffer (20 mM MES, 10 mM NaCl, 5 mM EDTA and 2 mM PMSF at pH 5.7), is loaded in the upward direction. The target proteins are adsorbed onto the SP resin while cell debris, cells, and unbound proteins pass through the column. Once the homogenate is loaded, the column is washed with 10 to 15 cv of equilibration buffer in the upward direction until the eluent is visually clear of debris. At this point the flow is reversed allowing the adsorbent particles to quickly settle. The target proteins (Hc fragment) are desorbed by step elution using 400 mM NaCl, 20 mM MES, 5 mM EDTA, 2 mM PMSF, pH 5.7 buffer. After the product is eluted the column is cleaned with 0.5 N NaOH + 1 M NaCl in the upward direction at 300 cm/h. The presence of H_c fragment in the samples is determined by clarifying a sample by centrifugation and loading the supernatant onto to a Poros HS column. The elution peak is then assayed by SDS-PAGE.

Results and Discussion

Initial binding studies of the H_c fragment on the Streamline column were performed using 40 mM acetate buffer at pH 5.6. Based on the SDS-PAGE there was very little product in the flow through fraction as compared to the load (data not shown). Unfortunately, it was soon noticed that the product pool was very unstable and protein precipitated during storage at 4°C. It was also observed that even after the homogenate

was clarified by centrifugation, a steady precipitation was observed in the supernatant fraction. Therefore, a study was conducted to determine protein stability in different buffers under different storage conditions. Table 2 shows the effect of buffer composition on protein stability under different storage conditions. Samples were frozen for 4 days prior to analysis. Samples on ice were stored for several hours. The optimum condition for minimal precipitation after homogenization is 20 mM MES, 150 mM NaCl at pH 5.7, unfortunately, the fragment will not bind to the Streamline column in 150 mM NaCl. Thus, it was necessary to reduce the NaCl content to 10 mM for the fragment to bind to Streamline column resin. It is evident that freezing the homogenate and processing later is not advisable because of the 31% loss caused by precipitation.

Table 2. The Effect of Buffer and Storage Conditions on the Protein Solubility of *P. pastoris* BoNT B Homogenates.

Buffer	Storage	Protein Loss (%)
40 mM Acetate Buffer, pH 5.6	Ice	43
40 mM Acetate Buffer, pH 5.6	Frozen	26
40 mM Acetate +150 mM NaCl, pH 5.6	Ice	40
40 mM Acetate +150 mM NaCl, pH 5.6	Frozen	26
20 mM MES + 10 mM NaCl, pH 5.7	Ice	13
20 mM MES + 10 mM NaCl pH 5.7	Frozen	31
20 mM MES + 150 mM NaCl pH 5.7	Ice	7
20 mM MES + 150 mM NaCl pH 5.7	Frozen	37

Several loading studies were conducted on the Streamline column. The amount loaded onto the column had an effect on the total amount of protein recovered. Loading studies were based on the total amount of protein loaded onto the column and not the amount that bound to the column. Under the conditions investigated only 3 to 5% of the total protein loaded onto the column was recovered in the elution peak. Approximately 95% of the soluble protein along with all of the debris flowed through the column. The yield from different loading studies are presented in Table 3 for a 73 mL column.

Table 3. StreamLine Column Loading Study with *P. pastoris* BoNT B Homogenates.

Total Protein Loaded (mg)	Column Load (mg/ml resin)	Total Protein Recovered (mg)	Percent Total Protein (%)
800	11	27	3.4
4000	55	186	4.7
9000	123	330	3.7

Based on the preliminary results

there appears to be an optimum protein load for the best percent recovery of total protein. The data are preliminary and studies are in progress to determine the reproducibility of these results. The Streamline column is capable of recovering, on average, 3.9 % of the total protein and the product pool is approximately 80% H_c fragment based on SDS-PAGE, Figure 3. The fragment was not detected in the flow thru. The Streamline resin has been cleaned and reused 5 times with out any change in performance.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Materials and Methods

Toyopearl 7.5 x 85 mm MD-P columns were pre-packed with 35 μm Toyopearl 650S, butyl, phenyl, or ether (TosoHaas, Montgomeryville, PA). Columns were run on a Waters HPLC (Millipore, Milford, MA) or Varian Model 5000 Liquid Chromatograph (Varian, Walnut Creek, CA). Absorbance at 280 nm was monitored with either a Waters 484 UV/Vis detector or a Varian UV100 detector and chromatograms were plotted and integrated by either a Spectra-Physics model 4270 integrator (Spectra-Physics, San Jose, CA) or Chromperfect software (Justice Innovations, Mountain View, CA) interfaced with a personal computer. Nearly all screening was performed at a flow rate of 1.5 mL/min (204 cm/hr) for the MD columns. The protein load for most of the screening runs was 100 μg to 500 μg of material from the Poros HS chromatography step.

Results and Discussion

Screening of Hydrophobic Interaction Chromatography Methods

Hydrophobic interaction chromatography (HIC) was tested as a potential process step in the purification of the BoNT B (Hc). Several different HIC resins were evaluated, including ether, phenyl and butyl columns. A variety of buffers, salts and gradient conditions were examined. Table 4 summarizes the results of the HIC method screening. The loading salt concentration ranged from 0.6 M to 1.2 M ammonium sulfate (the product precipitates at 1.6 M) and 1.5 M sodium chloride, which was required to bind the product to the column. The butyl column was preferred because elution of the product peak was at a lower salt concentration, while still providing adequate retention of the

product. The slope of the elution gradient had a significant effect on the resolution, with a steeper gradient (about 10 column volumes) showing the best overall results.

Standard Protocol

After examining the results of the screening runs, the Toyopearl butyl-650S resin with ammonium sulfate in 50 mM Na MES buffer pH 6.5 was determined to give the best results. The chromatographic conditions are shown in Table 5.

Table 4. HIC Screening Conditions

Column Type	Salt	Buffer	Comments
Ether-650S	$(NH_4)_2SO_4$	Na MES pH 5.5	Single peak, elutes at high salt.
Phenyl-650S	$(NH_4)_2SO_4$	Tris Cl pH 8.5	$(NH_4)_2SO_4$ is unstable at this pH.
Phenyl-650S	$(NH_4)_2SO_4$	Na PO_4 pH 7.0	Resolution of 65 kD impurity
Phenyl-650S	$(NH_4)_2SO_4$	Na MES pH 6.5	Resolution of 65 kD impurity.
Phenyl-650S	$(NH_4)_2SO_4$	Na Citrate pH 4.5	Single peak, high background.
Butyl-650S	$(NH_4)_2SO_4$	Na MES pH 6.5	Resolution of 65 kD impurity.
Butyl-650S	$(NH_4)_2SO_4$	Na MES pH 6.5, 5% EtOH	Poor Resolution
Butyl-650S	$(NH_4)_2SO_4$	Na PO_4 pH 7.0	Resolution of 65 kD impurity.
Butyl-650S	NaCl	Na MES pH 6.5	Poor resolution
Butyl-650S	NaCl	Na PO_4 pH 7.0	Poor resolution
Butyl-650S	NaCl	Tris Cl pH 8.5	Poor resolution

Under these conditions the Toyopearl butyl-650S column resolved Poros HS product pool into three or four protein peaks, Figure 4. The first peak elutes in the void volume. The second peak consists of a 65 kD impurity as seen on SDS-PAGE (Fig. 5) and elutes at approximately 0.3 M $(NH_4)_2SO_4$. It does not usually show baseline separation from the third peak, which contains greater than 90% of the total protein. When the HS product pool degrades substantially, a shoulder or even a separate unresolved

Table 5. Conditions for HIC on Toyopearl Butyl 650S.

Step	Conditions	Column Volumes
Equilibration	0.75 M $(NH_4)_2SO_4$ in 50 mM Na MES, pH 6.5	10
Loading	Sample in 0.75 M $(NH_4)_2SO_4$ in 50 mM Na MES, pH 6.5	Variable
Wash	0.75 M $(NH_4)_2SO_4$ in 50 mM Na MES, pH 6.5	5
Elution	Linear Gradient to 0 M $(NH_4)_2SO_4$	10
Wash	0 M $(NH_4)_2SO_4$ in 50 mM Na MES, pH 6.5	5

peak (47 kD fragment) appears on the leading edge of the main product peak. Because of the overlap of this peak with the main product peak, the 47 kD fragment is normally present in all but the trailing edge of the main product peak, based on SDS-PAGE.

Column Geometry

The effect of column length on resolution was examined. A 4.6 x 250 cm column was packed with ToyoPearl butyl-650S and run under equivalent conditions as the MD columns, Figure 6. The longer column resulted in greater resolution of the 65 kD impurity from the main product peak, but no significant improvement in resolving the 47 kD impurity from the product peak.

Sample Preparation

In order to load the HS product pool onto a HIC column, a buffer exchange step is required. After the Poros HS step, the product pool is in 20 mM Na MES, pH 5.7 with approximately 150 mM NaCl and must be exchanged into 20 mM NaMES, pH 5.7 with 0.75 M $(NH_4)_2SO_4$. An Amicon S1Y10 spiral cartridge was used to diafilter the HS product pool. During diafiltration there was always some precipitation. Analysis by SDS-PAGE showed that the product was precipitating. Protein loss was estimated at 13% by protein assay.

After buffer exchange degradation of the H_c fragment to the 47 kD fragment occurred. HS product pool was dialyzed over night (18 h) at 4°C into 0.75 M $(NH_4)_2SO_4$, 20 mM MES pH 6.5 and stored at 4°C after dialysis. As seen in Figure 7, the front shoulder of the main product peak increased between injections. Chromatogram A was immediately after dialysis and chromatogram B is 3 h after the dialysis, while chromatogram C is 24 after dialysis. Peak 2 corresponds to the 47 kD fragment, Figure 8. Such rapid degradation of the product was unexpected and has not been seen before. At this point the mode of degradation is unknown. It could be a function of pH as the HS product pool is at pH 5.7 and the HIC starting buffer is pH 6.5. It is not know if ammonium sulfate caused destablization of the H_c fragment. Based on these results, diafiltration was used to reduce the amount of time (1 to 2 h) necessary to exchange the buffer from 20 mM MES, pH 5.7 to 20 mM MES pH 6.

Scale Up

The HIC procedure was scaled up to a 1x10 cm column with a protein load of 18 mg or 2.25 mg/mL of resin. Resolution remained satisfactory with this column, but after subsequent size exclusion chromatography the quality of the product was poor, with multiple bands seen on SDS-PAGE (not shown).

Conclusions

The HIC purification step with Toyopearl Butyl-650S resin resulted in some purification of the main product peak, most notably removal of the proteins present in the flow through peak and the 65 kD impurity. There was a general perception that some of the minor low molecular weight impurities were removed HIC, but the intensities of these bands were often difficult to judge by SDS-PAGE. In previous purification protocols the impurity at 65 kD was removed by size exclusion chromatography and is probably removed more effectively than by HIC. Because of the inability of HIC to remove the 47 kD fragment, the HIC step was eliminated from further consideration.

POROS PI CHROMATOGRAPHY

Material and Methods

Poros 20 PI

The column was a 4.6 x 100 mm (1.66 ml) Poros 20 PI column (Perseptive Biosystems) operated at 7 ml/min (2527 cm/hr) with injection volumes of 5 ml unless otherwise noted. The PI column was evaluated with different buffers at varying pHs and the methods are listed below according to the buffers.

Ethanolamine (EA) buffer

The column equilibration buffer was 25mM ethanolamine (EA) pH 8.5. The buffer was made online by the BioCad by mixing nanopure water with 100 mM EA, pH 8.5. The sample, which was a fraction from the Poros HS column, was dialyzed overnight into 10mM EA, pH 8.5. The column load was 0.2 mg of protein/ml resin.

Protein was eluted using a linear gradient from 25 to 60mM EA over 25 column volumes (CV), followed by a salt wash from 0-200mM NaCl over 5 CV.

Diethanolamine (DEA) buffer

Samples (HS fraction) were dialyzed to 25mM DEA, pH 8.5 and protein load onto the column was 0.3 to 0.4 mg/ml resin. Elution gradient conditions were the same as described for the EA buffer system.

Tris-HCl buffer

A gradient of Tris buffer, pH 8.5, from 50 - 200 mM over 25 cv was attempted. Samples were dialyzed in either 25 mM DEA or 25 mM Tris-HCl. Acetonitrile from 0 to 5% was added to the running buffer in an attempt to sharpen peaks.

pH gradient from 9.0 to 6.0 with Tris/Bis-Tris-Propane buffer

Tris/Bis-Tris-Propane (T-BTP) buffer was used over a pH range from 9 to 6. The pH gradients were mixed by the BioCad to 25mM or 15mM from 0.1M T-BTP, pH 9 and 0.1M T-BTP, pH 6. The pH gradient was over 25 cv.

Poros 50 PI

A series of experiments were conducted with different column sizes and linear velocities using several of the buffer systems. The recommended superficial linear velocity for the 50 μm PI is 1000 cm/hr. A 0.46 x 10 cm (1.66 ml) column was evaluated at 2.77 ml/min (1000 cm/hr), a 0.46 x 25 cm (4.15 ml) column was evaluated at 3 ml/min (1083 cm/hr), a 1.6 x 14 cm (28ml) was tested at 17 ml/min (507 cm/hr) and a 5.0 x 9 cm (177 ml) at 40 ml/min (122 cm/h). A linear velocity of 122 cm/h was maximum permissible because of system limitations. It is expected that the Poros 50 PI will be operated at 500 to 1000 cm/h.

pH step gradient

A step gradient was implemented to simplify the process at a large scale. The column was equilibrated with 15mM T-BTP, pH 8.5 subsequent pH steps of 8.0, 7.0, and 6.0 at constant 15 mM T-BTP, followed by a gradient from 0-500mM NaCl. Samples

were dialyzed into 20mM Tris-HCl, pH 8.7, conductivity 0.5mS (final sample pH, 8.6-8.7; final conductivity, 0.6mS).

Results and Discussion

The first buffer system that was tested was ethanolamine at 25 mM, pH 8.5. The elution profile with EA was not reproducible and was probably caused by the poor buffering capacity of EA at pH 8.5 and 25 mM. The reproducibility of the chromatograms improved when the pH increased to 9.5, however resolution did not, Figure 9. In addition, there was concern about protein stability at such a high pH. From these experiments it was determined that it was possible to bind the protein at pH 8.5, though inconsistently. These experiments served as a starting point for screening different buffers and conditions.

The next buffer evaluated was diethanolamine (DEA), which has a pK_a of 8.8. DEA proved to be a better buffer than EA at pH 8.5. Analysis of the peaks (Figure 10) by SDS-PAGE (Figure 11) indicated that the conditions were producing some level of separation of the 53 kD band from the 47 kD band. It was apparent that the method was separating out more proteins than expected. Peak 4, which elutes in the NaCl gradient, has multiple bands from 53 kD to 47 kD suggesting that the 53 kD band that elutes during the linear gradient of DEA may be different than the 53 kD band that elutes in the NaCl gradient. Peak 1 is only partially retarded and does not completely bind to the column. Comparison of the chromatogram with EA at pH 9.5, Figure 9, indicates that all of the protein was bound to the column indicating that complete binding is possible under proper conditions.

The DEA buffer system generated a reproducible process at the analytical scale and was able to partially separate the 47 kD protein from the 53 kD main peak, but the separation was not adequate at this point and a different buffer system was evaluated.

Tris-HCl was evaluated at pH 8.5 with a linear buffer gradient from 25 to 200 mM Tris-HCl with the sample dialyzed into 25 mM DEA, pH 8.5, Figure 12. When the sample was dialyzed into 25 mM Tris and loaded, part of the sample did not bind to the column (Figure 13). It appears that the type of buffer used for sample loading has an

effect on binding and the fact that part of the material did not completely bind indicates that overall binding is not very strong.

The Tris-HCl gradient partially resolved the degradation fragment (47 kD) from the 53 kD band. The degradation fragment was resolved from the 53 kD band on the leading edge of the second peak (fraction 19-23), but not on the trailing (Figure 14). For samples that were dialyzed in Tris-HCl, some of the 53 kD material eluted before the start of the gradient. Analysis of the third and fourth peak by SDS-PAGE suggested different species at the same general molecular weight. Based on the SDS-PAGE analysis and behavior of the material on the PI column there appears to be several different species that elute at approximately 53 kD.

The next step was to investigate a pH gradient to elute the proteins. A Tris/Bis-Tris-Propane (T/BTP) buffer system over a pH range of 9.0 to 6.0 was tested. A buffer concentration of 15 mM T/BTP and a pH gradient from 8.5 to 6.0 resulted in a clean 53 kD product peak, however there was still not complete binding of the protein to the column with elution prior to start of pH gradient (Fig. 15). This may have been caused by the sample being dialyzed into 20 mM Tris, pH 8.6, which does not buffer as well as T/BTP. During the gradient the pH was held for 6 column volumes at pH 8.0. The 2 major peaks, which eluted during the pH gradient, both had a 53kD band (Figure 16), but had different banding patterns on a pH 5-8 IEF (Figure 17). The pH gradient was able to generate a pure product by separating the 47 kD protein from the 53 kD product protein and the other 53 kD proteins that elute later in the gradient.

Based on the results from pH gradient studies it was decided to attempt a step pH gradient. The optimum conditions were to equilibrate the column with 15 mM T/BTP at pH 8.5 to 8.6 and a conductivity of 0.65 to 0.7 mS. Samples were buffer exchanged into15 mM T/BTP, pH 8.8 to 9.0 at a conductivity of 0.6 mS. The load pH was increased to completely bind the protein based on studies of binding efficiency at several pH's and conductivities (data not shown) and the binding that was seen at pH 9.5 with EA. It is necessary that the conductivity of the load material be less than or equal to the conductivity of the column equilibration buffer to facilitate complete binding. The first

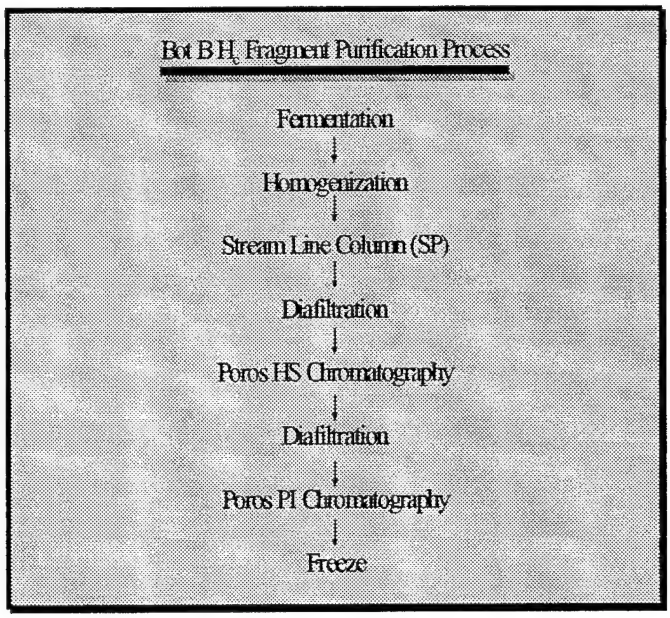
elution step was 15 mM T/BTP at pH 8.0, next was 15 mM T/BTP pH 7.0, followed by a salt gradient at pH 7.0 from 0 to 500 mM. Initially, the BioCad was programmed to blend the step pH gradient and incomplete binding was observed. When each buffer was prepared individually there was complete binding of the material.

The process was successfully scaled-up to the 50 μm resin in a 1.6 cm by 14 cm (28 ml) column at a protein load of 0.44mg/ml resin (Figure 18). Using T/BTP for each elution step resulted in complete binding to the column and sharp distinct peaks. Under these conditions there was no 47 kD fragment in the main 53 kD peak. Analysis of the fractions by IEF showed that Peak 1 is primarily one band at a pI of 7.3, while peak 2 contained the 47 kD fragment and a 53 kD band by SDS-PAGE (data not shown) and had a completely different banding pattern on IEF than Peak 1, (Figure 19).

The PI step was able purify the BoNT B product to 99%+, unfortunately the buffers in this step are not USP. To meet GMP, it is preferred to have the last several process steps use USP buffers. Unfortunately, we found that T/BTP, which is not USP, was the best buffer to bind the protein to the column. As an alternative, it was decided to determine if the product could be eluted in a USP buffer. The strategy was to equilibrate and load the column with T/BTP and then wash the column with Tris-HCl (USP) at the same pH and conductivity. After a certain number of column volumes of Tris-HCl were flushed through the column to displace the T/BTP, the product was eluted with pH 8.0 Tris-HCl (USP). The column was eluted further with T/BTP because of the superior buffering capacity at pH 7.0. This procedure was used at the next largest scale, 5.0 cm by 9 cm (177 mL column). A chromatogram of the process is presented in Figure 20. The product profile on SDS-PAGE and IEF were identical to previous results indicating that buffer exchange is possible on the column.

PURIFICATION PROCESS

The outline of the purification process for BoNT B H_c fragment is presented below. The process has three chromatography steps. The first process step is disruption of the *Pichia* to release the H_c fragment. The homogenate is then loaded onto a Streamline column, washed and eluted with 20 mM MES, pH 5.7 and 400 mM NaCl. The product pool from the Streamline is diafiltered with 20 mM MES, pH 5.7 using an Amicon 10,000 MWCO regenerated cellulose spiral ultrafiltration membrane (YM10). The diafiltration is stopped when the solution conductivity reaches that of the Poros HS column equilibration buffer. The Streamline pool is loaded onto the HS column at 1 mg of total protein/mg resin and eluted using a NaCl gradient from 10 to 150 mM. The HS product pool, which elutes at approximately 130 mM is pH adjusted to pH 8.8 with 1 M T/BTP. The pH adjusted HS product pool is then diafiltered with 15 mM T/BTP, pH 8.8 until the conductivity is 0.6 to 0.65 mS. The material is filtered through a 0.45 μm filter and loaded onto a Poros PI column at concentration of 0.5 mg of total protein/ml of resin. The product pool is eluted using a step pH gradient. The product pool from the PI column is then frozen at -80°C in the elution buffer.



Exhibition Lot 12/12/96

An exhibition run of the process was tested at a small scale. The exhibition lot was based on 150 g of wet cell mass for the homogenization step in a final volume of 2 L. The column size for each step is listed in Table 6.

A complete spreadsheet description of each one of the steps is found in the Appendix. All of the details of the process, including buffer types and volumes, column size and a listing of raw materials are presented in the spreadsheets. A summary of the exhibition run is presented in Table 7. SDS-PAGE and IEF for each of the steps in the process are presented in Figures 21 and 22, respectively. The PI step produces final product which is a single band on SDS-PAGE and nearly a single band on pH 5 to 8 IEF. The yield of the PI step is 56%, with 93% of the total protein load accounted for. Based on the IEF gel there was no product present in any of the other peaks that eluted from the PI column (Figure 22).

Table 6. Column Sizes for Exhibition Lot 12/12/96

Step	Column Type	Column Size (ml)	Column Dimension i.d. (cm) x length (cm)
2	Streamline SP	73	2.5 x 15
4	Poros HS	80	2.6 x 15
6	Poros PI	177	5.0 x 9.0

The yield of final product from 150 g of wet cell mass was 59 mg. A liter of fermentation broth will produce typically 200 g of wt cell mass, thus resulting in about 79 mg, while a 60 L fermentation would produce about 4.7 g of final purified product using the existing process.

There are several steps in the process that require further optimization to increase overall yield. As mention above, though not reflected in Table 7, is the 50%-60% efficiency of the homogenization step. It is reasonable to expect the disruption efficiency to increase from 60% to 95%. Next, the yield of the Poros HS step is 39.4%. Preliminary studies indicate that a majority of the protein is precipitating on the column, and studies are underway to increase the yield to an expected value of 65% to 70%. Also, the HS diafiltration step yield is 81.2% while the PI diafiltration step is 96.6%. A yield of 90 to 95% for each diafiltration step is expected.

FUTURE PLANS

It is necessary to perform more detailed protein load and recovery studies on the Streamline, Poros HS and PI steps. This will include maximum protein load per mg of resin, as well as determining the maximum protein concentration of the load. Flow rate

studies to determine the optimum flowrate for loading and elution are also planned. Stability of pure product in Tris, pH 8.0 is currently under study, and other USP approved buffers will be evaluated to determine the best storage buffer and temperature. Finally, studies will be conducted to determine the expected protein recovery during the diafiltration steps.

PROJECT CONCLUSIONS TO DATE

At this point a scaleable process has been developed for the fermentation and purification of the BoNT B H_c fragment. The product is 99+% pure based on SDS-PAGE using a 8 μg load stained with coomassie blue. The product also appears to be 97 to 98% pure based on IEF from pH 5 to pH 8.0. IEF is certainly one of the toughest criteria for purity as it is possible to have different isoforms (charge modifications to different amino acids) that can cause different bands to appear. The purification process for Bot B will be further improved, but at this point a major landmark in the project has been achieved. This process, both fermentation and purification, has been transferred to Walter Reed as of December 11, 1996.

REFERENCES

1. M. Meagher, 1996. Final Report of Army Project DAMD17-95-C-5003.

FIGURES

Figure 1. Methanol feed rate and cell density (OD_{600}) during induction phase of BotNB H_c fermentation.

Figure 2. Western blot of PEI and $(NH_4)_2SO_4$ treated homogenate samples taken during the MeOH induction of the BotNB H_c fermentation. Time of induction is indicated above each lane.

Figure 3. Streamline fractions of BotNB H_c in 20 mM MES, pH 5.7. Lane 1 is molecular weight markers. Lane 2 is cell homogenate. Lane 3 Streamline flow through. Lane 4 is Streamline product fraction. Lane 5 is Poros HS product fraction.

Figure 4. HIC chromatogram of HS product pool using a 7.5 by 85mm Toyopearl butyl-650S column. Peak 1 is flow through. Peak 2 is 65 kD protein. Peak 3 is primarily H_c fragment.

Figure 5. Chromatogram of HS product pool using a 4.6mm x 25 cm Toyopearl 650S butyl column. Peak identification is the same as Figure 4.

Figure 6. SDS-PAGE (10 % gel) of fractions of the HS product pool separated on a the 4.6 mm x 25 cm Toyopearl 650S butyl column. Gel was stained by coomassie blue.

Figure 7. Chromatograms showing degradation of starting material in 0.75 M Ammonium sulfate, 50 mM Na MES, pH 6.5, after 18 h of dialysis. Chromatogram A is 0 h after dialysis. Chromatogram B is 3 h after dialysis and Chromatogram C is 24 h after dialysis. Samples were maintained at 4°C.

Figure 8. SDS-PAGE (10% gel) of load material, Peak 2 and Peak 3 from Figure 7.

Figure 9. Chromatogram of HS product pool with PI column using 25 mM ethanolamine, pH 9.5. The gradient was from 25 mM EA to 200 mM EA over 25 column volumes.

Figure 10. Chromatogram of HS product pool using 25mM diethanolamine, pH 8.5 as the equilibration buffer. The gradient was from 25 mM to 200 mM DEA.

Figure 11. SDS-PAGE (8-16% gradient gel) of fractions from Figure 10. Gel was stained with coomassie blue.

Figure 12. Tris-HCl Chromatogram of PI column with sample dialyzed into DEA buffer.

Figure 13. Tris-HCl chromatogram of PI column with sample dialyzed into Tris-HCl buffer.

Figure 14. SDS-PAGE (8-16% gradient gel) of fractions from Figure 13. Gel was stained with coomassie blue.

Figure 15. Tris/Bis-Tris-Propane chromatogram with a gradient 8.5 to 6 with the pH held at 8.0 for 6 column volumes before resuming the pH gradient. The sample was dialyzed into 20 mM Tris, pH 8.6.

Figure 16. SDS-PAGE (10% gel) of fractions from Figure 15. Gel was stained with coomassie blue.

Figure 17. Isoelectric focusing (IEF) of fractions from Figure 15 using a Pharmacia Phast Systems and pH 5-8 gels. Sample load volume is 4 µl and gels were silver stained using the Pharmacia silver stain kit.

Figure 18. Chromatogram of 28 ml PI column equilibrated in 15 mM T/BTP, pH 8.5. Product was eluted from the column using a step elution of T/BTP at pH 8.0 and 7.0, followed by a salt gradient. Sample was dialyzed into 15 mM T/BTP, pH 8.8.

Figure 19. IEF of Figure 19. Conditions are the same as outlined in Figure 17.

Figure 20. Chromatogram of 177 ml PI column of Exhibition run #1. The process conditions are described in the text.

Figure 21. SDS-PAGE (10% gel) of exhibition run #1. Gel was stained with coomassie blue.

Figure 22. IEF at pH 5-8 of exhibition run #1. Conditions are the same as outlined in Figure 17.

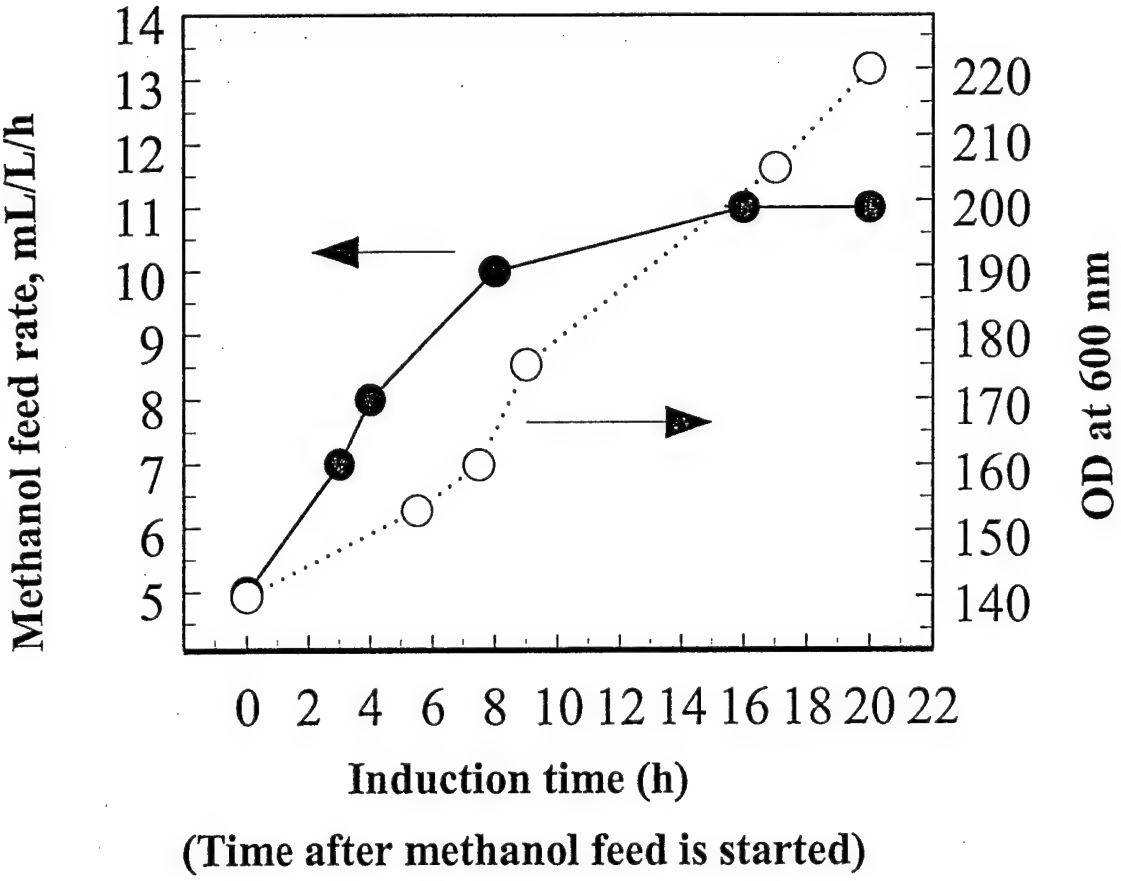

Methanol feed rate, mL/L/h
14
13
12
11
10
9
8
7
6
5
220
210
200
190
180
170
160
150
140
OD at 600 nm
0 2 4 6 8 10 12 14 16 18 20 22
Induction time (h)
(Time after methanol feed is started)

Fig. 1

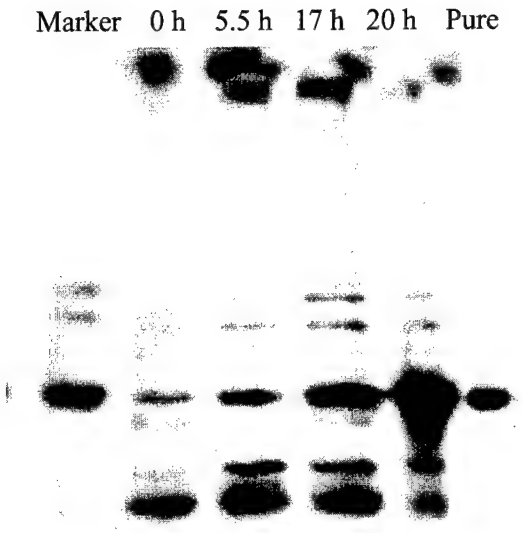


Fig. 2.

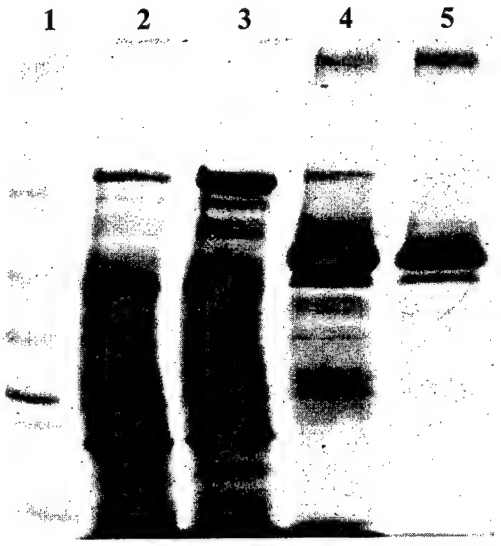


Fig. 3

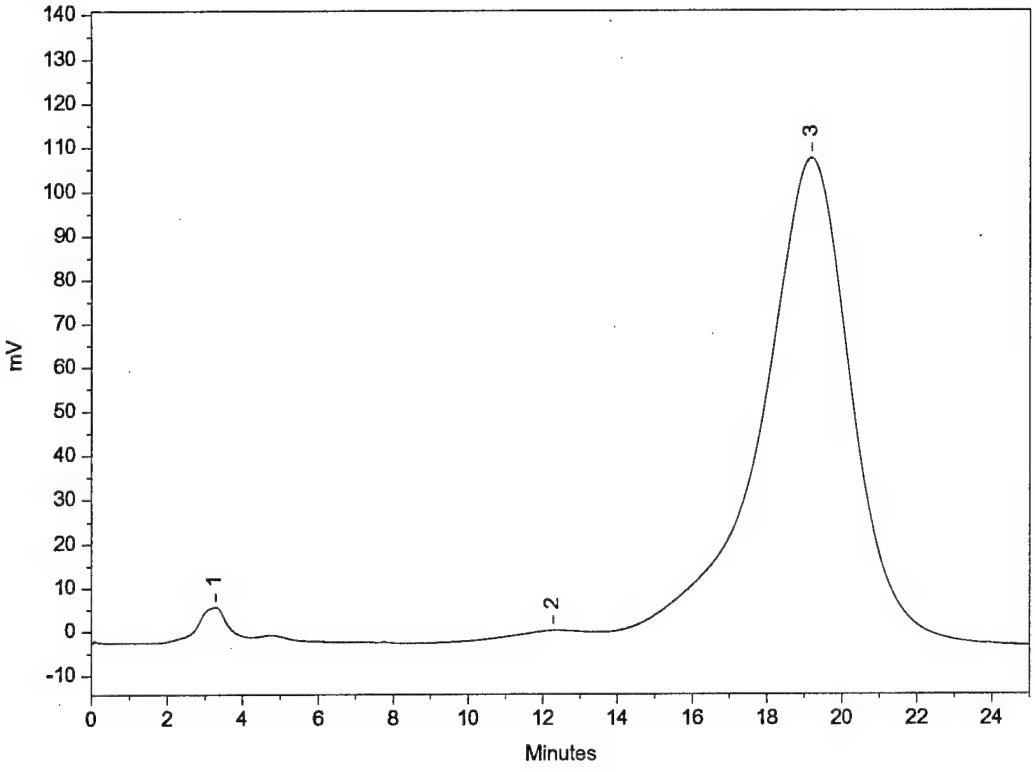

140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
mV
0
2
4
6
8
10
12
14
16
18
20
22
24
Minutes
1
2
3

Figure 4.

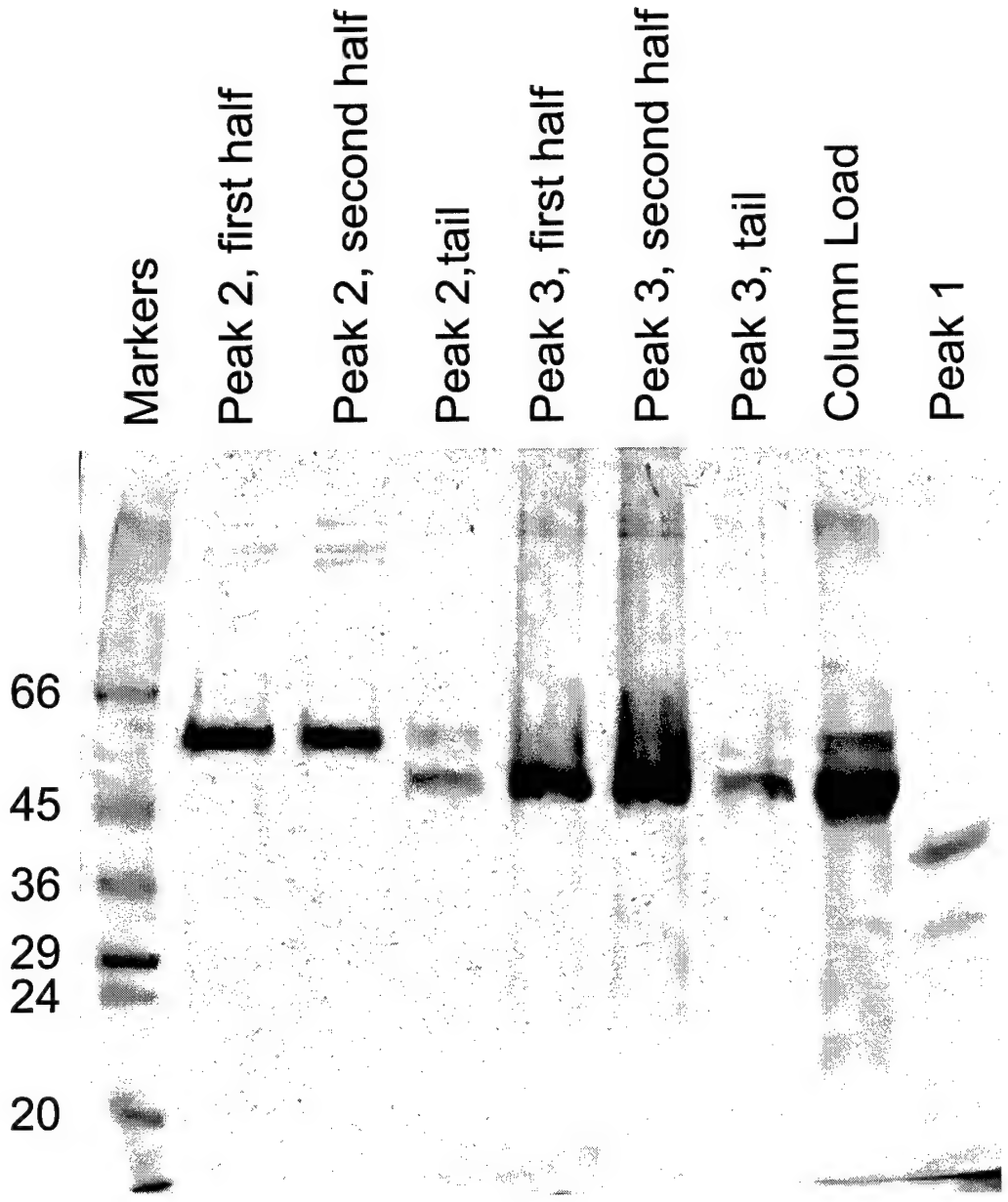

Markers
Peak 2, first half
Peak 2, second half
Peak 2,tail
Peak 3, first half
Peak 3, second half
Peak 3, tail
Column Load
Peak 1
66
45
36
29
24
20

Figure 5.

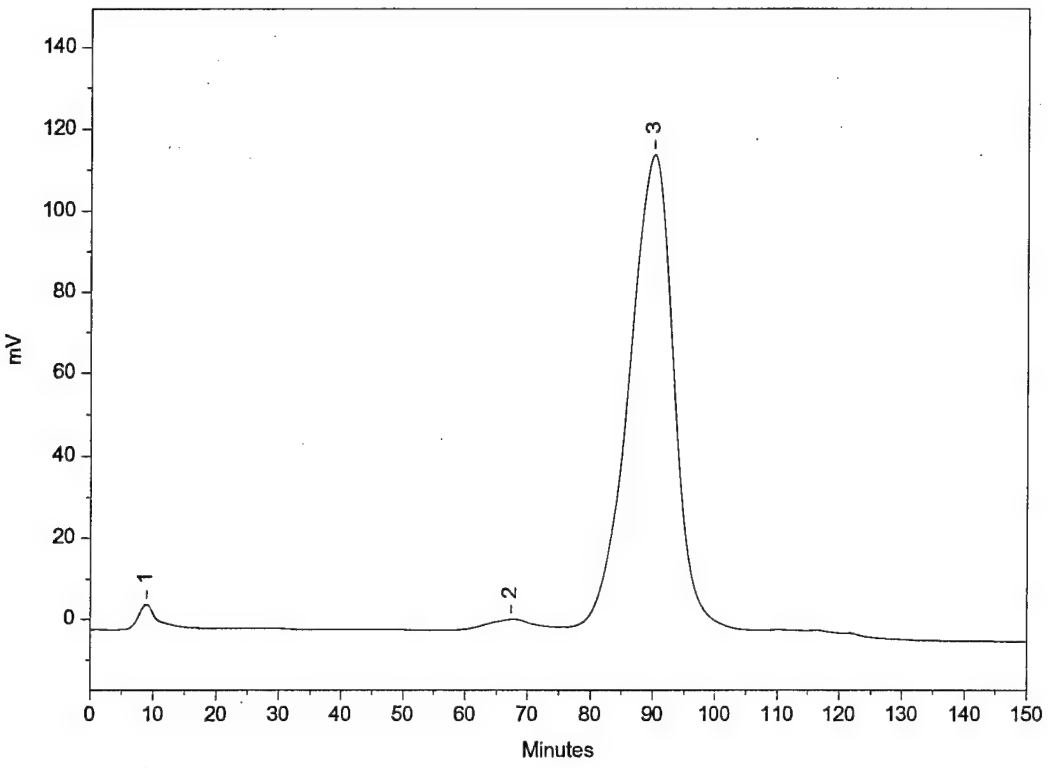

140
120
100
80
60
40
20
0
mV
1
2
3
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
Minutes

Figure 6.

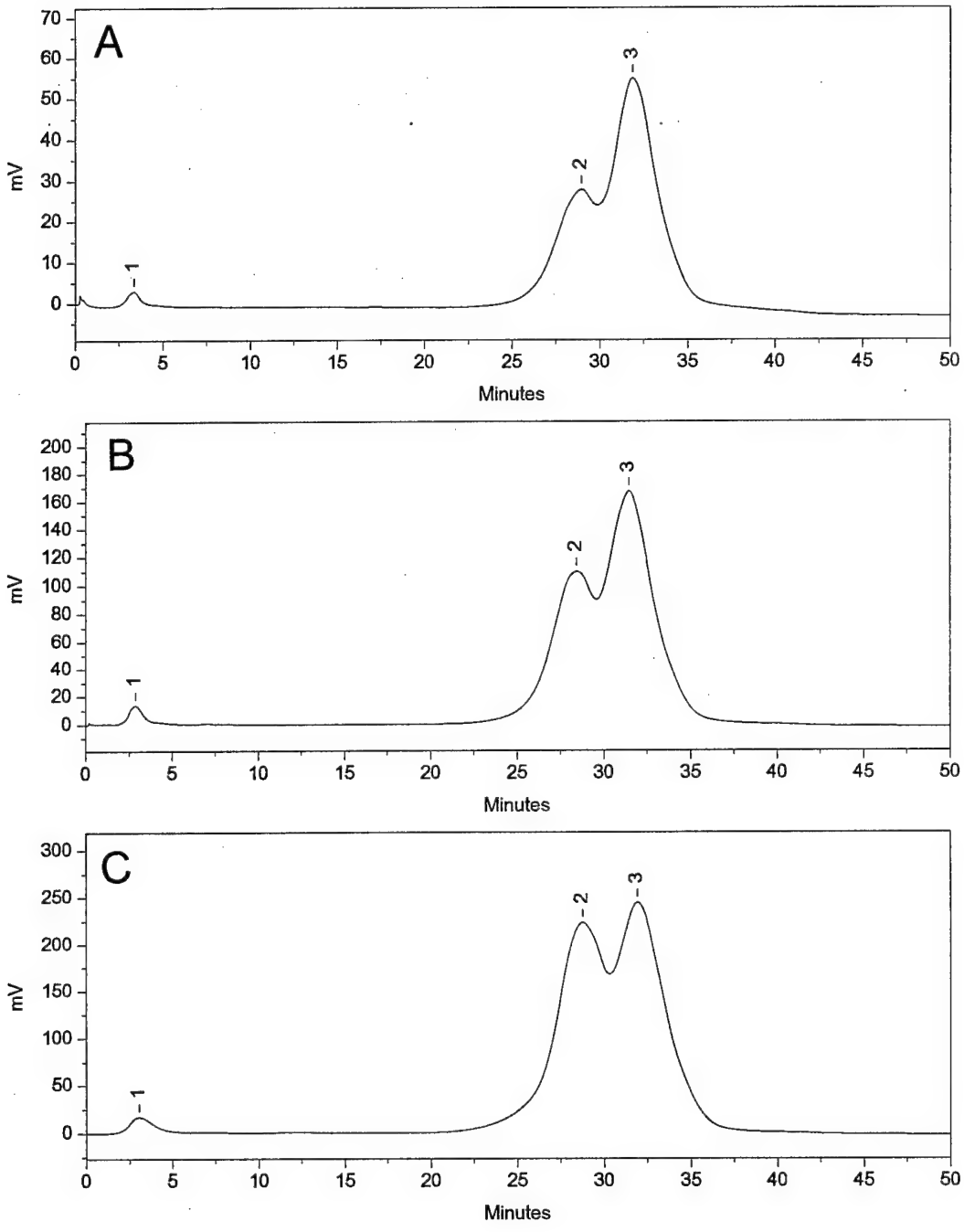


Figure 7.

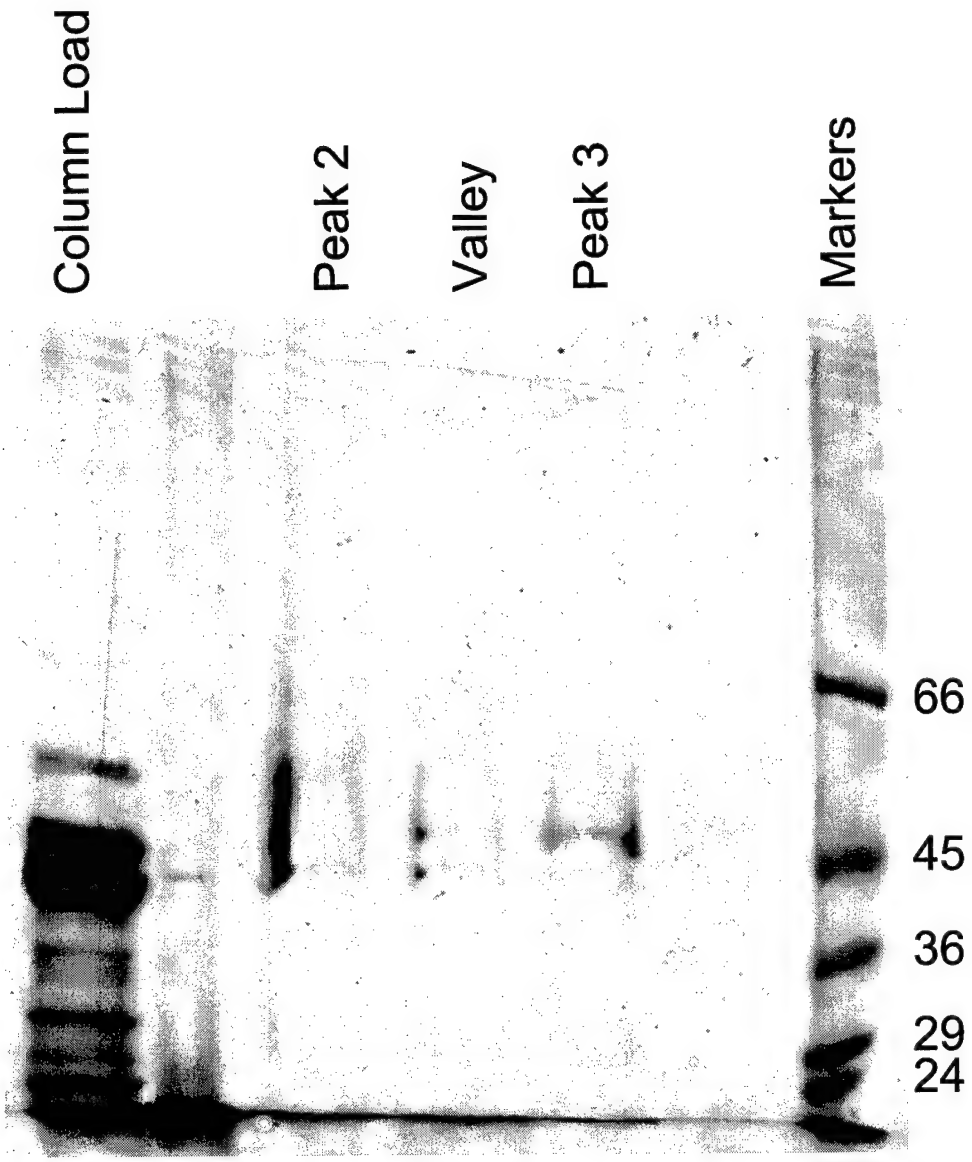


Figure 8.

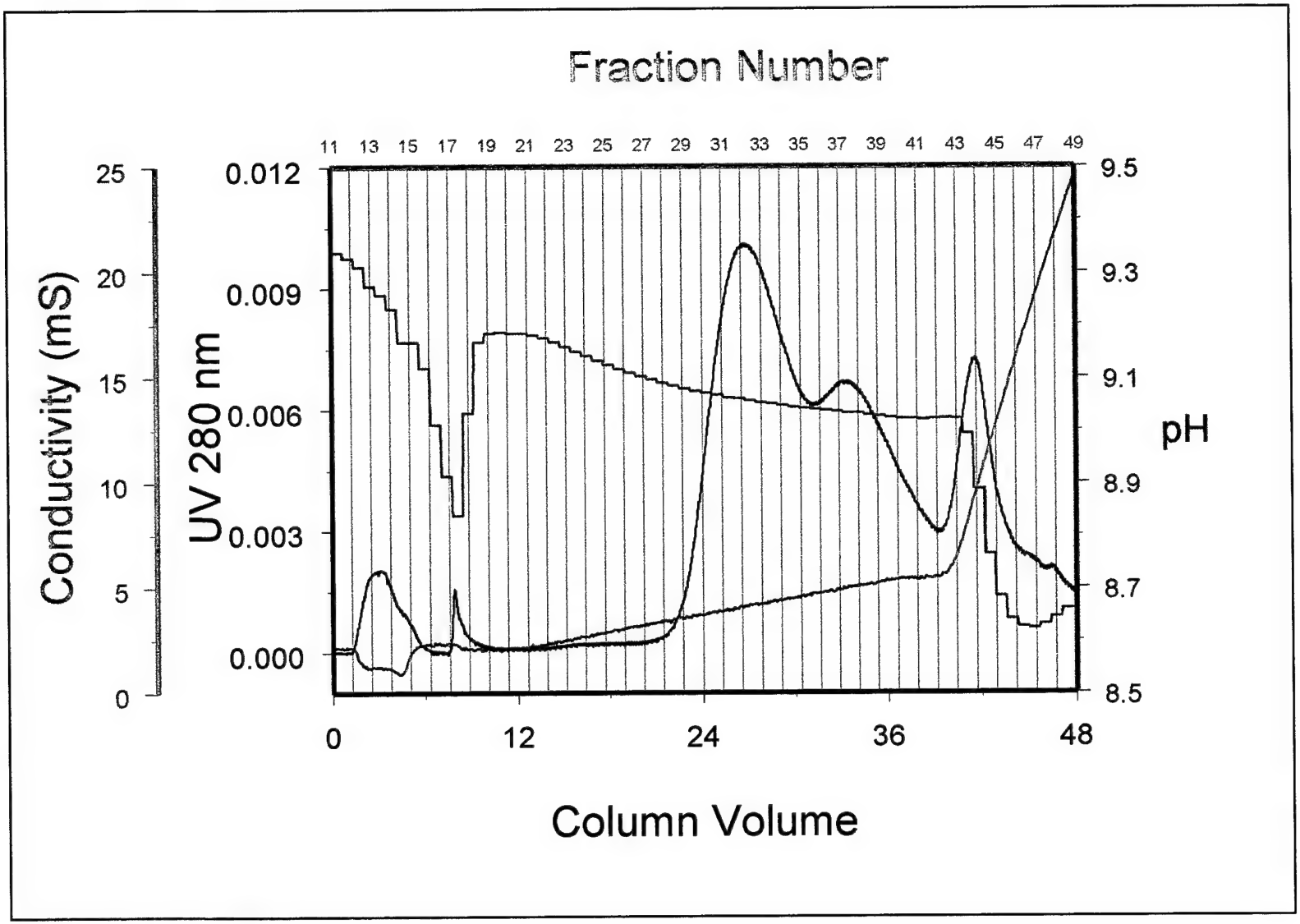

Fraction Number
11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49
Conductivity (mS)
25
20
15
10
5
0
UV 280 nm
0.012
0.009
0.006
0.003
0.000
pH
9.5
9.3
9.1
8.9
8.7
8.5
0
12
24
36
48
Column Volume

Figure 9

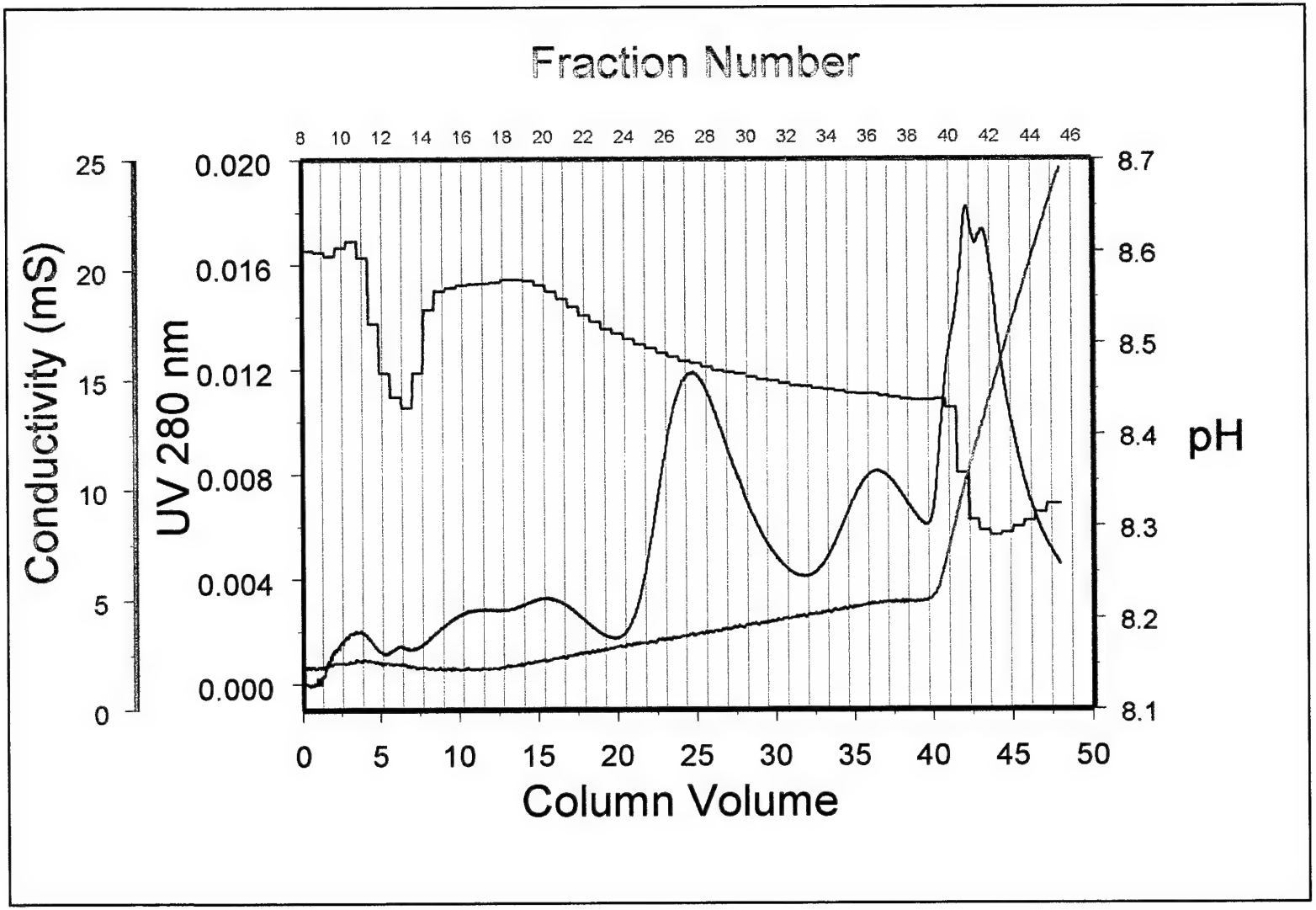

Fraction Number
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
Conductivity (mS)
25 20 15 10 5 0
UV 280 nm
0.020 0.016 0.012 0.008 0.004 0.000
pH
8.7 8.6 8.5 8.4 8.3 8.2 8.1
0 5 10 15 20 25 30 35 40 45 50
Column Volume

Figure 10

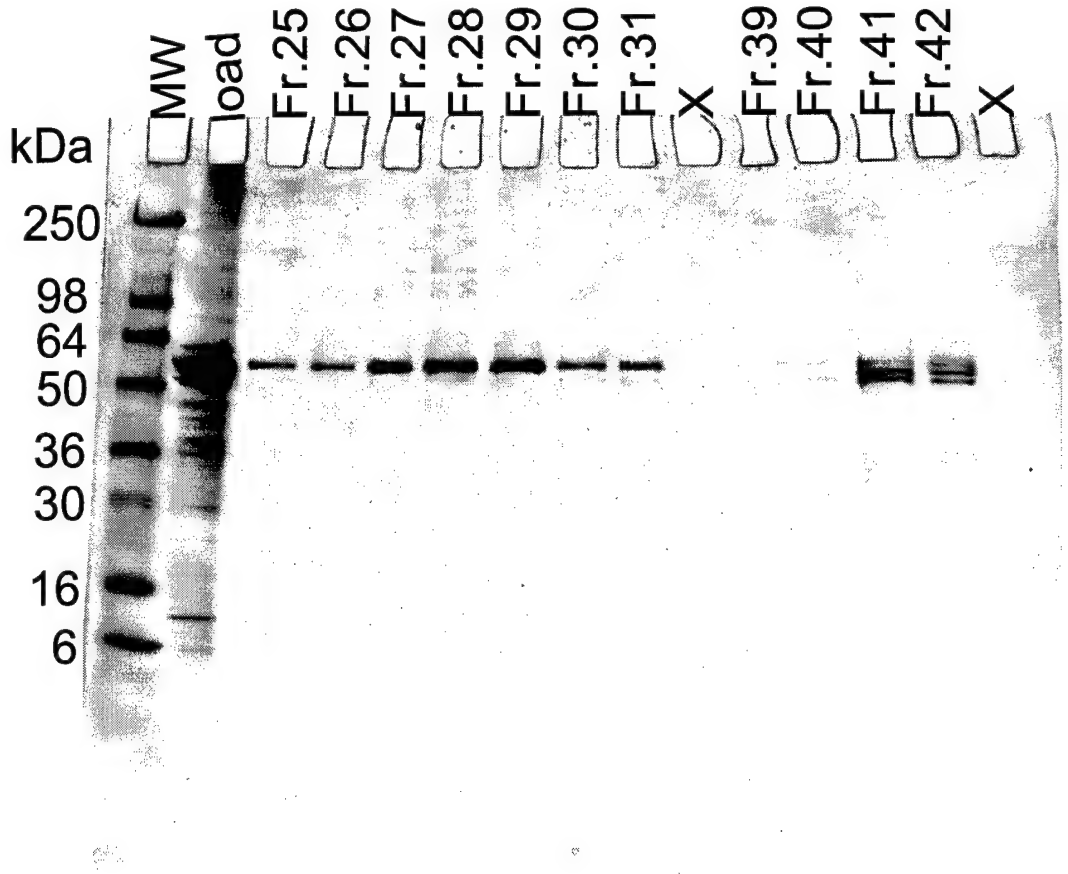

kDa
MW
load
Fr.25
Fr.26
Fr.27
Fr.28
Fr.29
Fr.30
Fr.31
X
Fr.39
Fr.40
Fr.41
Fr.42
X
250
98
64
50
36
30
16
6

Figure 11

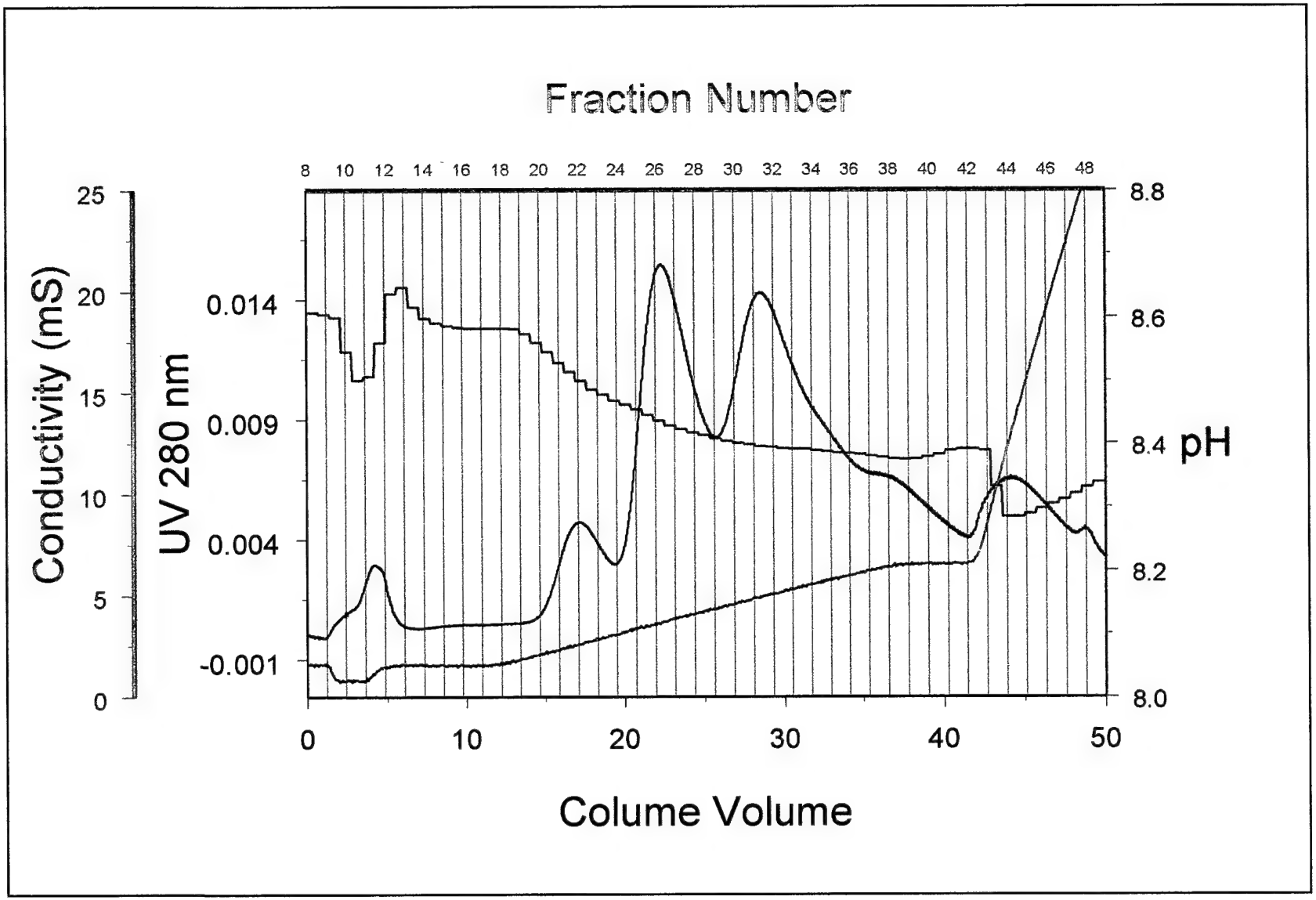

Fraction Number
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Conductivity (mS)
25
20
15
10
5
0
UV 280 nm
0.014
0.009
0.004
-0.001
pH
8.8
8.6
8.4
8.2
8.0
0
10
20
30
40
50
Colume Volume

Figure 12

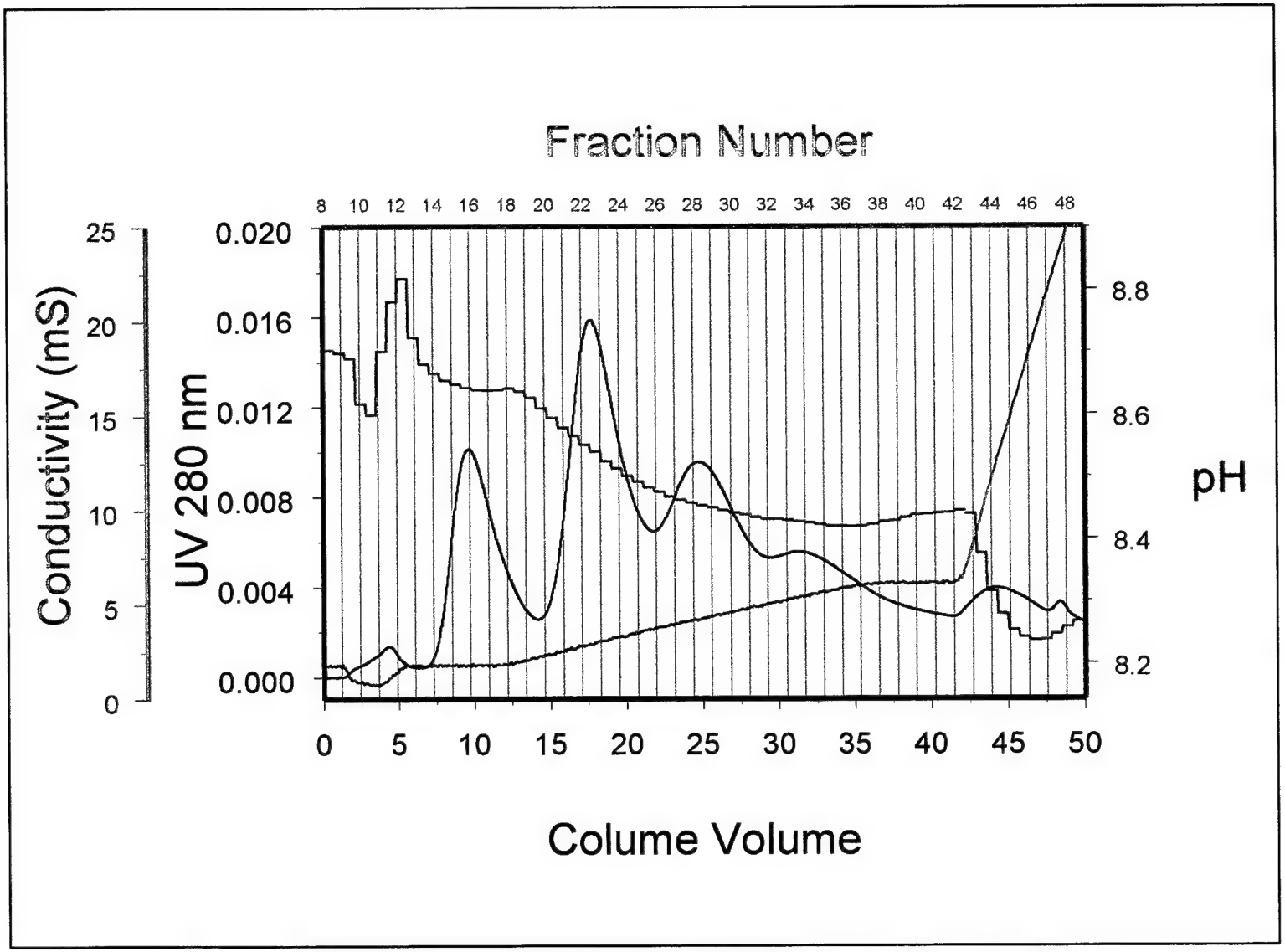

Fraction Number
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Conductivity (mS)
25 20 15 10 5 0
UV 280 nm
0.020 0.016 0.012 0.008 0.004 0.000
pH
8.8 8.6 8.4 8.2
0 5 10 15 20 25 30 35 40 45 50
Colume Volume

Figure 13

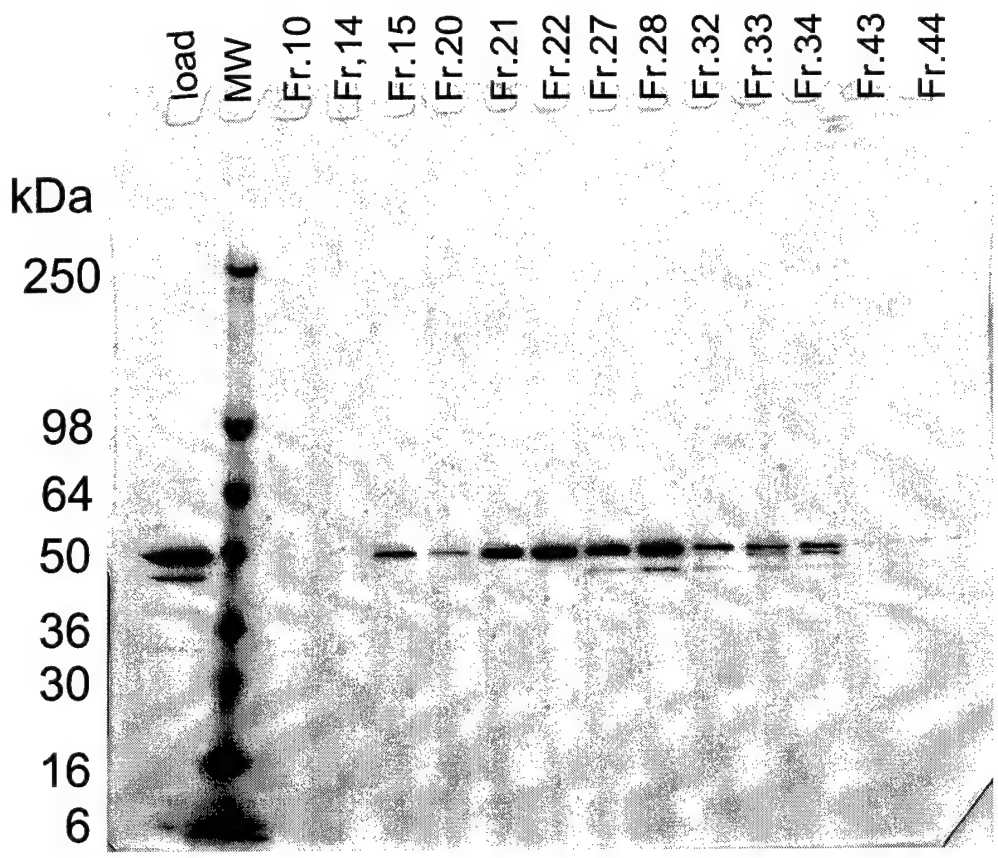

load
MW
Fr.10
Fr,14
Fr.15
Fr.20
Fr.21
Fr.22
Fr.27
Fr.28
Fr.32
Fr.33
Fr.34
Fr.43
Fr.44
kDa
250
98
64
50
36
30
16
6

Figure 14

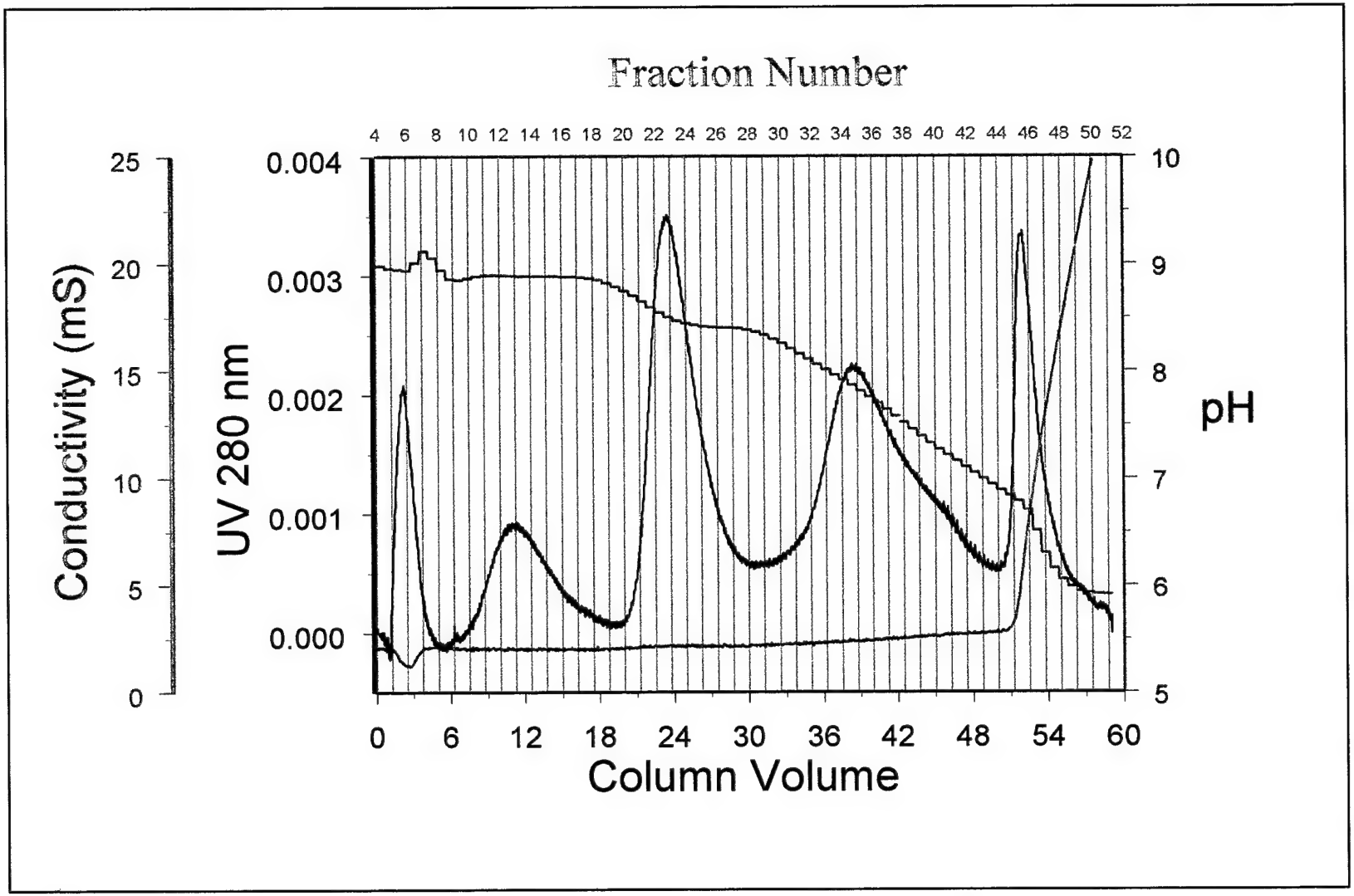

Fraction Number
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52
Conductivity (mS)
25
20
15
10
5
0
UV 280 nm
0.004
0.003
0.002
0.001
0.000
pH
10
9
8
7
6
5
0 6 12 18 24 30 36 42 48 54 60
Column Volume

Figure 15

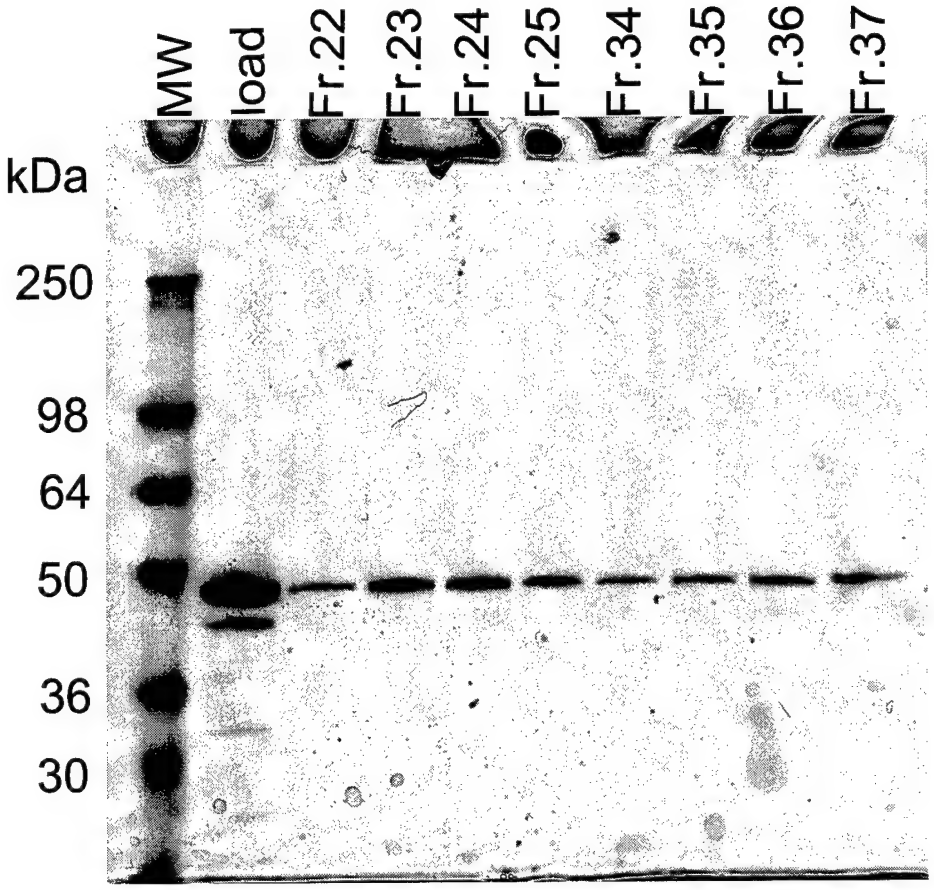

MW
load
Fr.22
Fr.23
Fr.24
Fr.25
Fr.34
Fr.35
Fr.36
Fr.37
kDa
250
98
64
50
36
30

Figure 16

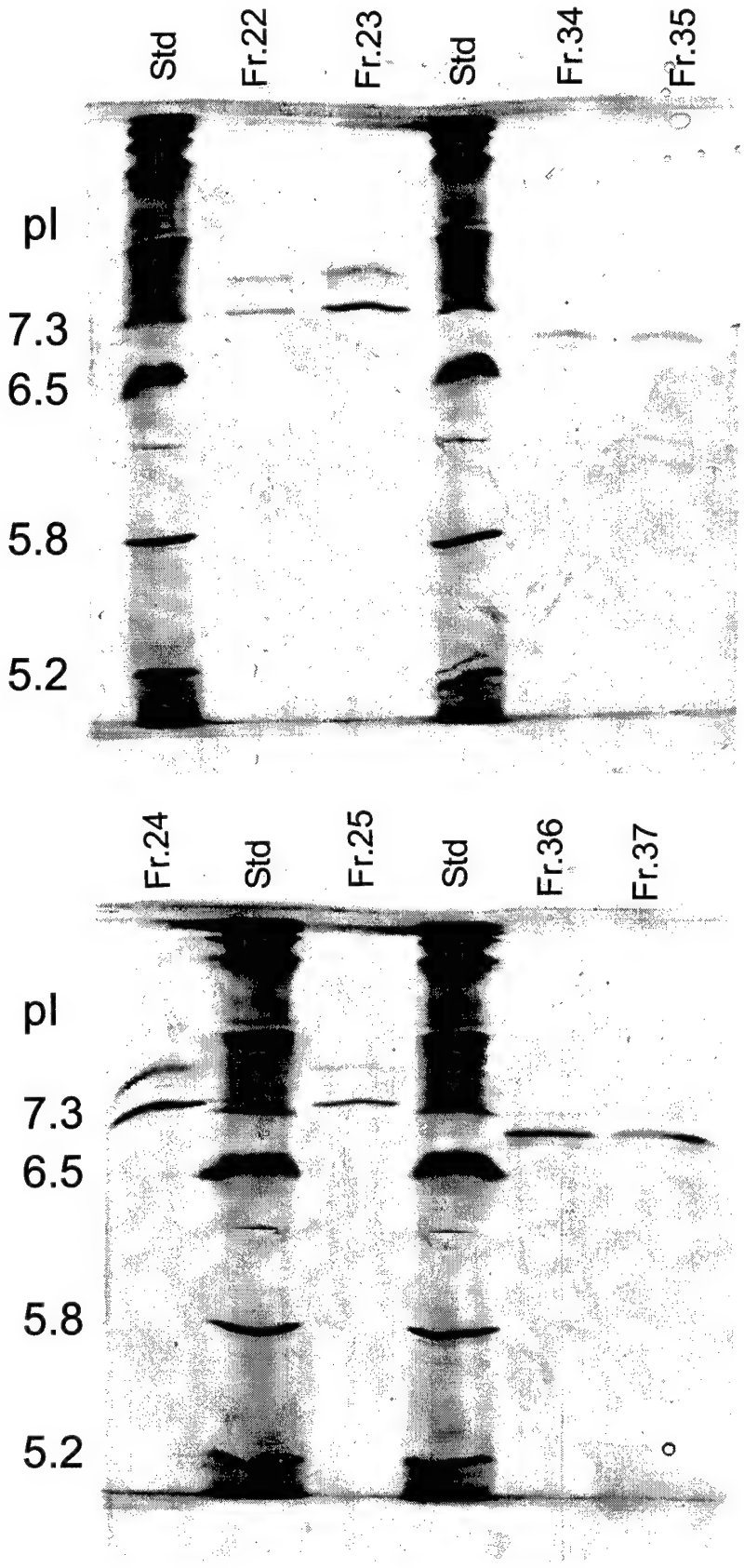

Std
Fr.22
Fr.23
Std
Fr.34
Fr.35
pI
7.3
6.5
5.8
5.2
Fr.24
Std
Fr.25
Std
Fr.36
Fr.37
pI
7.3
6.5
5.8
5.2

Figure 17

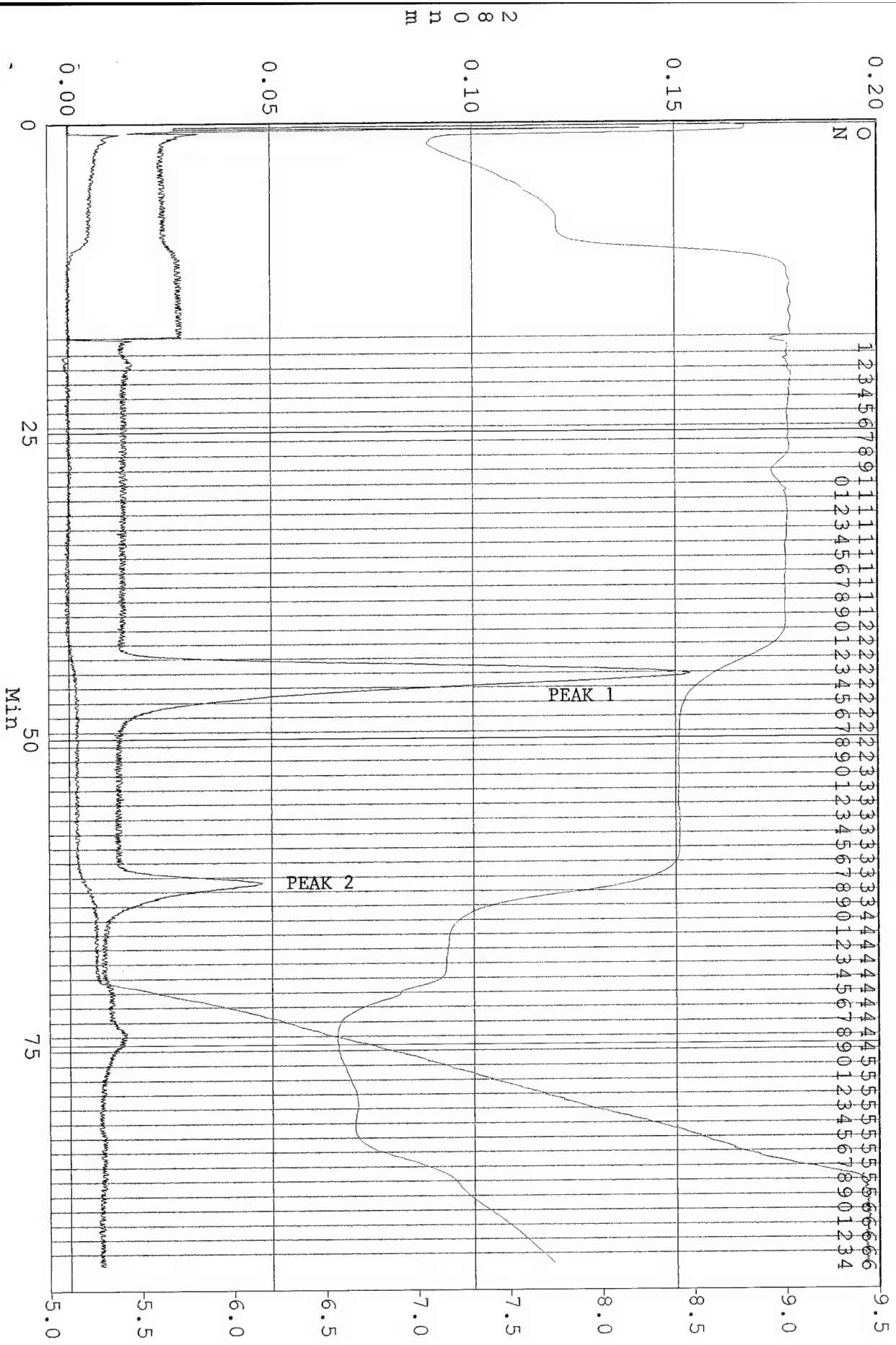


Figure 18

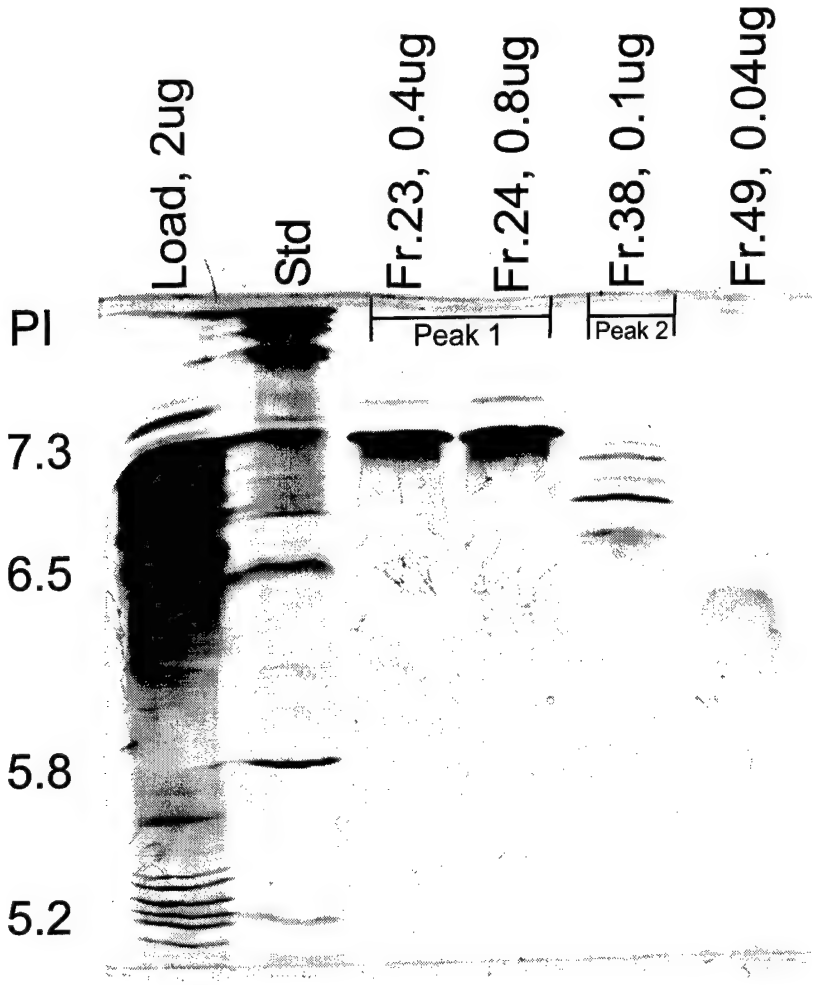
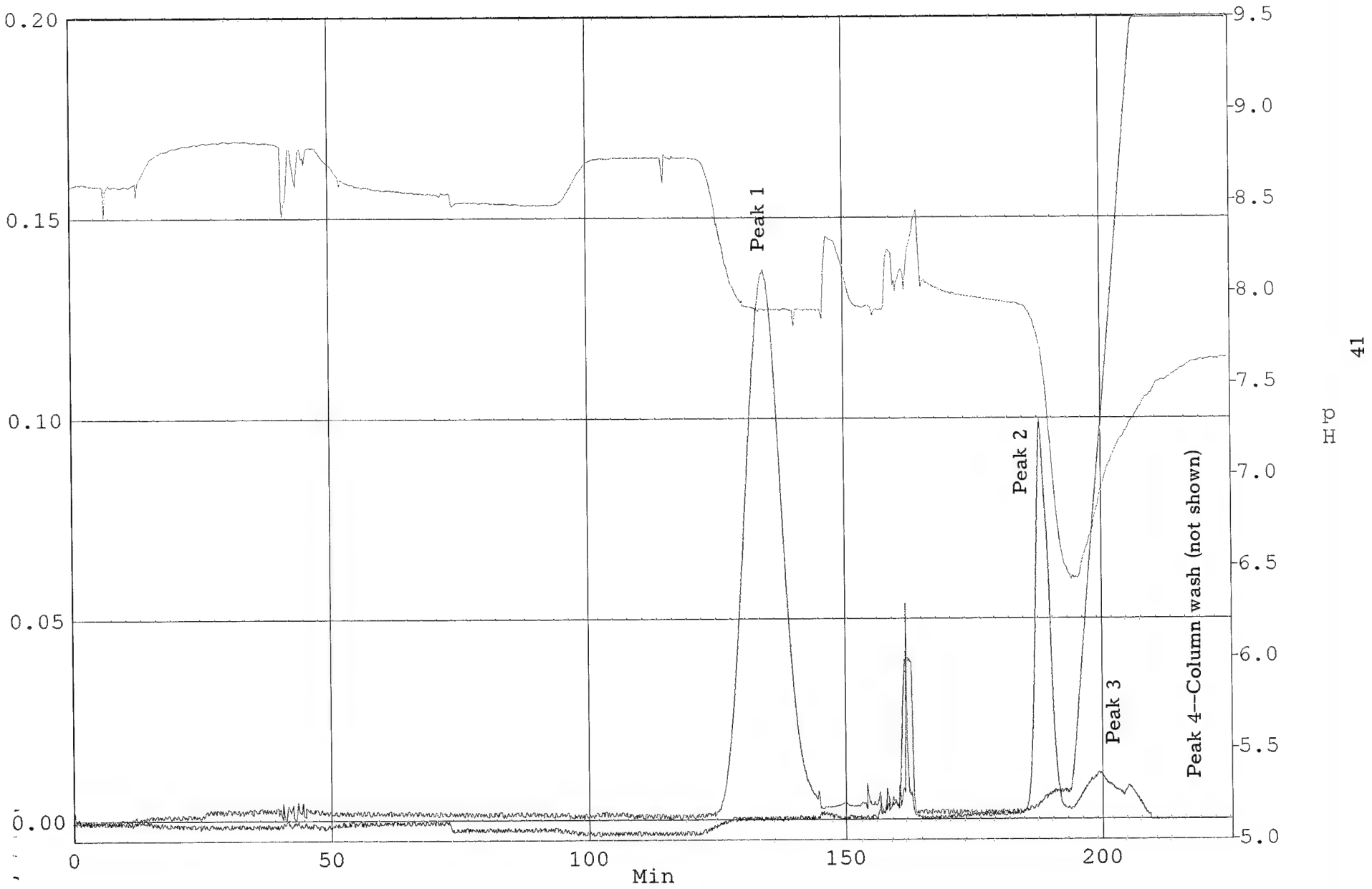

Load, 2ug
Std
Fr.23, 0.4ug
Fr.24, 0.8ug
Fr.38, 0.1ug
Fr.49, 0.04ug
PI
7.3
6.5
5.8
5.2
Peak 1
Peak 2

Figure 19

Figure 20


0.20
0.15
0.10
0.05
0.00
280nm
0
50
100
150
200
Min
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
pH
Peak 1
Peak 2
Peak 3
Peak 4--Column wash (not shown)

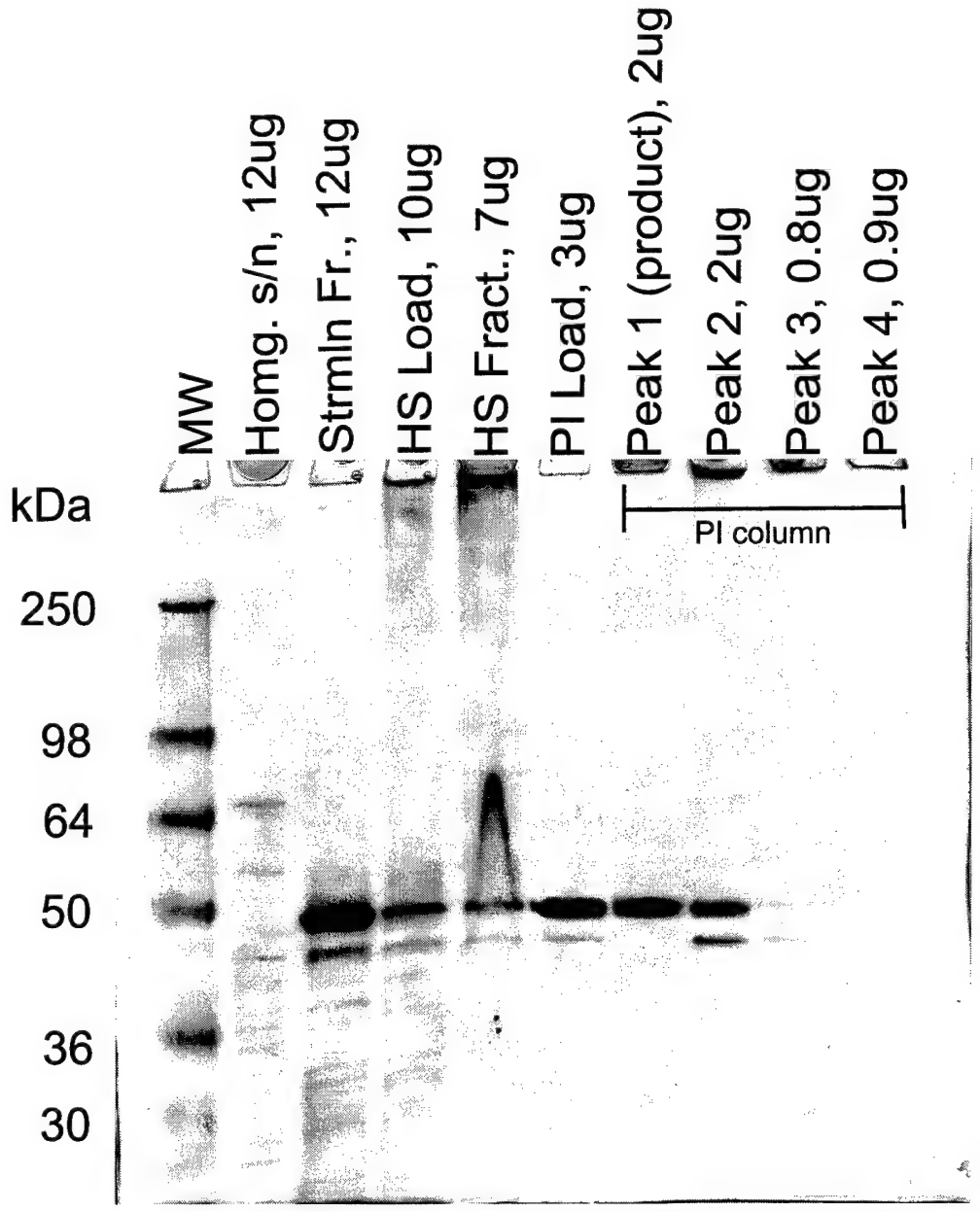
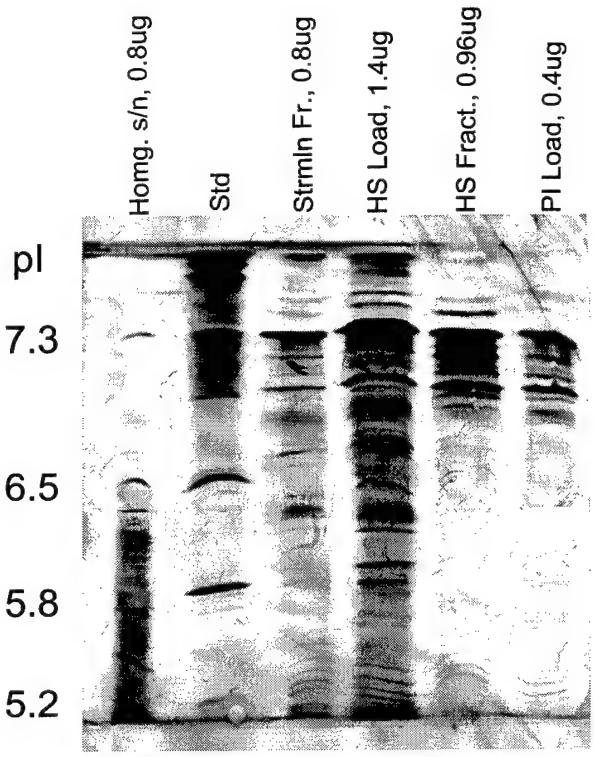
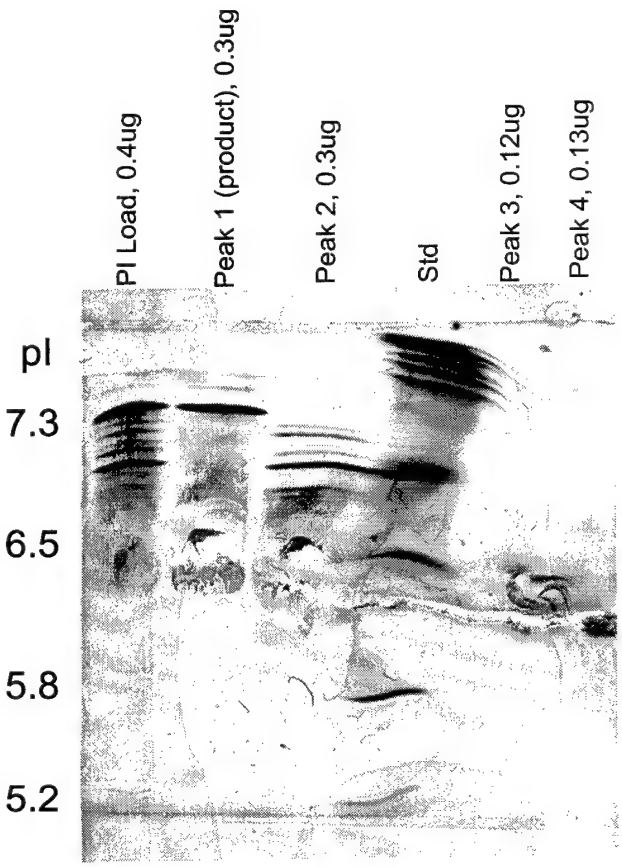

MW
Homg. s/n, 12ug
Strmln Fr., 12ug
HS Load, 10ug
HS Fract., 7ug
PI Load, 3ug
Peak 1 (product), 2ug
Peak 2, 2ug
Peak 3, 0.8ug
Peak 4, 0.9ug
PI column
kDa
250
98
64
50
36
30

Figure 21


Homg. s/n, 0.8ug
Std
Strmln Fr., 0.8ug
HS Load, 1.4ug
HS Fract., 0.96ug
PI Load, 0.4ug
pI
7.3
6.5
5.8
5.2


PI Load, 0.4ug
Peak 1 (product), 0.3ug
Peak 2, 0.3ug
Std
Peak 3, 0.12ug
Peak 4, 0.13ug
pI
7.3
6.5
5.8
5.2

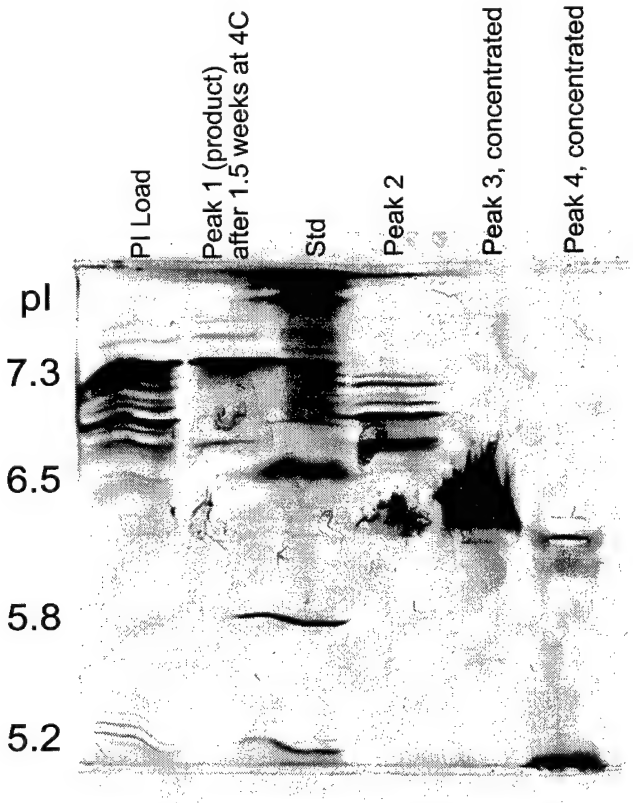

PI Load
Peak 1 (product) after 1.5 weeks at 4C
Std
Peak 2
Peak 3, concentrated
Peak 4, concentrated
pI
7.3
6.5
5.8
5.2

Figure 22

APPENDIX

Homogenization — Step No.: 1

Process Information

Homogenization Buffer	g/l
20 mM MES (2-(N-Morpholino)ethanesulfonic acid	4.26
5 N NaOH to pH 5.7	
5 mM EDTA (Ethylenediaminetetraacetic acid)	1.46
2 mM PMSF (Phenylmethylsulfonyl flouride) **(Note)**	0.38
Batch Size	3 kg
Buffer Requirements	30 L

Protein Assay

The Bradford protein assay is used to determine the amount of soluble protein released during disruption. Samples are centrifuged using a microfuge and assayed using the BioRad Protein Assay.

Raw Material Requirements

Item	g
MES (2-(N-Morpholino)ethanesulfonic acid	127.8
5 N NaOH to pH 5.7	
5 mM EDTA (Ethylenediaminetetraacetic acid)	43.8
2 mM PMSF (Phenylmethylsulfonyl flouride)	11.52

Process Description

Process Description: Suspend 100 g wet cells/L of homogenization buffer. The solution should be kept at 4 C prior to homogenization. Prepare the solution just prior to homogenization. The solution is homogenized at a pressure of 19,000 psig or higher but no greater than 25,000 psig. Multiple passes will be required to disrupt the cells. Chill the homogenate immediately to 4 C prior to the next pass. A minimum of 4 g/l soluble protein must be released. This corresponds to 50% disruption. A disruption efficiency of 95% will result in a soluble protein concentration of 8 g/L.

Note: PMSF is made as a stock solution. The composition is 100 mM (19 g/L) in 100% ethanol and added to the buffer solution to a final concentration of 2 mM.

Stream Line Column — Step No.: 2

Required Information

Media:	SP	Eluting Linear Velocity (cm/h):	100.00
Column Diameter (cm):	20.00	Load Protein Concentration (mg/ml):	4.00
Packed Column Height (cm):	20.00	Col. Loading Cap. (total mg protein/ml resin):	21.00
Total Column Height (cm):	85.00	Acutal Column Binding Capacity (mg/ml):	1.11
Loading Linear Velocity (cm/h):	200.00		

Calculated Parameters

Column Volume (L):	6.28	Loading Flow Rate (ml/min):	1,047
Expanded Resin Bed Height (cm):	40.00	Elution Flow Rate (ml/min):	524
Total Load Volume (L):	32.99		
Total Protein Loaded (g):	131.95		
Total Protein Eluted From Column (g):	7.00		

Column Buffers

Equilibration Buffer	g/L
20 mM MES (2-(N-Morpholino)ethanesulfonic acid	4.26
5 N NaOH to pH 5.7	
10 mM NaCl (Sodium chloride)	0.58
5 mM EDTA (Ethylenediaminetetraacetic acid)	1.46
2 mM PMSF (Phenylmethylsulfonyl flouride) **(Note)**	0.38
Elution Buffer	g/L
20 mM MES	4.26
5 N NaOH to pH 5.7	
5 mM EDTA	1.46
2 mM PMSF	0.38
400 mM NaCl	23.38
Regeneration Buffer	g/L
1 N NaOH	40.00
1 M NaCl	58.40

Buffer Requirements Per Cycle

Buffer	**Volume (L)**
Equilibration Buffer	428.83
Elution Buffer	31.42
Regeneration Buffer	133.52

Raw Material Requirements Per Cycle

Item	**Amount (g)**
MES	1,827
5 N NaOH	
EDTA	626
PMSF	177
NaCl	985
NaOH	7,797

Note: PMSF is made as a stock solution. The composition is 100 mM (19 g/L) in 100% ethanol and added to the buffer solution to a final concentration of 2 mM.

Process

Step	Buffer/Description	Mode	Col. Vol. (CV)	Total Vol. (L)
1	Equil. Buf./Equilibrate Column	Upflow	3.0	80.11
2	Homogenate/Load Column	Upflow	1.2	32.99
3	Equil. Buf./Wash Column	Upflow	10	267.04
4	Equil. Buf./Pack and Wash Column	Downflow	3.0	18.85
5	Elution Buffer/Elute Column	Downflow	5.0	31.42
6	Regeneration Buf./Clean Column	Upflow	5.0	133.52
7	Equil. Buf./Reequilibrate column	Downflow	10.0	62.83

Process Description: Column is loaded in the fluidized state with cell homogenate. Column is washed with equilibration buffer in the upflow to wash cells and unbound material from the column. Once the column is free of particles the flow is reversed to pack the column. Eluent is monitored at UV280. Once baseline absorbance is zero step elute with elution buffer.

Note: Store column in 200 mM Na Acetate+ 20% by vol. EtOH.

Pre Poros HS Diafiltration Step — Step No.: 3

Process Information

Membrane Size:	10,000 MWCO
Membrane Type:	YM10
Membrane Chemistry:	Regenerated Cellulose
Membrane Manufacture:	Amicon
Membrane Configuration:	Spiral
Diafiltration Buffer:	20 mM MES pH 5.6

Retentate Inlet Pressure (psig):	40.00
Retentate Outlet Pressure (psig):	30.00
Maximum Protein Concentraton (mg/ml):	1.00
Liner velocity (m/s):	0.50
Flux Rate (LMH):	30.00
Diafiltration Volumes:	4.00
Operating Temperature (C):	8.00
Final Conductivity (mS):	Same as the equilibration buffer for HS column
Final pH:	5.60

Process Description

Process Description: The purpose of this step is to reduce the NaCl concentration to 10 mM by diafiltration so that it can be loaded onto the Poros HS column. Typical diafiltration volumes required to reach the expected conductivity is approximately 5.

Poros HS Column — Step No.: 4

Required Information

Media:	50 um HS
Column Diameter (cm):	20.00
Column Height (cm):	10.00
Loading Linear Velocity (cm/h):	800.00
Eluting Linear Velocity (cm/h):	800.00
Load Protein Concentration (mg/ml):	1.00
Column Binding Capacity (mg/ml):	1.00
Column Yield (%):	60.00

Vendor Information

Chromatographic media is 50 micron HS Poros resin from Perseptive Biosystems.

Column and Sample Conditions

Column and elution buffers are at room temperature and the column load is kept at 4 C. The product peak is collected on ice and chilled to less than 10 C.

Calculated Parameters

Column Volume (ml):	3,142	Loading Flow Rate (ml/min):	4,189
Total Protein Loaded (mg):	3,142	Eluting Flow Rate (ml/min):	4,189
Total Load Volume (ml):	3,142	Total Protein Eluted From Column (mg):	1,885

Column Buffers

HS Equilibration Buffer (A)	**g/L**
20 mM MES (2-(N-Morpholino)ethanesulfonic acid	4.26
5 N NaOH to pH 5.7	
10 mM NaCl (Sodium chloride)	0.58
HS Elution Buffer (B)	**g/L**
20 mM MES	4.26
5 N NaOH to pH 5.7	
150 mM NaCl	8.76
HS Elution Buffer (C)	**g/L**
1 M NaCl	58.4
HS Cleaning Buffer (1)	**g/L**
1 N NaOH	40.00
1 M NaCl	58.40
HS Cleaning Buffer (2)	**g/L**
D.I. Water	1000
HS Cleaning Buffer (3)	**g/L**
1 M Acetic Acid	60

Buffer Requirements Per Cycle

Buffer	Volume (L)
HS Equilibration Buffer (A)	102.10
HS Elution Buffer (B)	64.40
HS Elution Buffer (C)	15.71
HS Cleaning Buffer (1)	15.71
HS Cleaning Buffer (2)	15.71
HS Cleaning Buffer (3)	15.71

Raw Material Requirements Per Cycle

Item	Amount (g)
MES	709.31
5 N NaOH	
NaCl	2458.48
NaOH	628.32
Acetic Acid (Glacial)	942.48
D.I. Water (L)	232.48
Total Processing Time (min)	**55.5**

Process

Step	Buffer/Description	C.V.	Total Vol. (L)
1	Equilibrate Column with HS Equilibration Buffer (A)	5.0	15.71
2	Load Column with StreamLine Pool	1.0	3.14
3	Wash Column with HS Equilibration Buffer (A)	5.0	15.71
4	Linear Gradient of 100% Buffer (A) to 100% Buffer (B)	35.0	109.96
5	Hold Gradient at 100% Buffer (B)	3.0	9.42
6	Step Elute with Elution Buffer (C)	5.0	15.71
7	Wash Column with Cleaning Buffer (1)	5.0	15.71
8	Wash Column with Cleaning Buffer (2)	5.0	15.71
9	Wash Column with Cleaning Buffer (3)	5.0	15.71
10	Equilibrate Column with HS Equilibration Buffer (A)	5.0	15.71
	Total	74.0	232.48

Process Description: Column is loaded, washed, and eluted with a linear gradient of sodium chloride from 10 mM to 150 mM with the main peak eluting at approximately 130 to 140 mM NaCl. The column is washed with regeneration buffer and reequilibrated with equilibration buffer. Detection is at UV 280 nm.

Note: Store column in 0.1 M Na Acetate+ 20% by vol. EtOH.

Pre Poros PI Diafiltration Step — Step No.: 5

Process Information

Membrane Size:	10,000 MWCO
Membrane Type:	YM10
Membrane Chemistry:	Regenerated Cellulose
Membrane Manufacture:	Amicon
Membrane Configuration:	Spiral
Diafiltration Buffer:	15 mM Tris-Bis-Tris Propane pH 8.8
Conductivity of Diafiltraiton Buffer (µs):	0.6
pH Adjustment Buffer:	1 M Tris-Bis-Tris-Propane pH 9.5

Retentate Inlet Pressure (psig):	40.00
Retentate Outlet Pressure (psig):	30.00
Maximum Protein Concentraton (mg/ml):	0.10
Liner velocity (m/s):	0.50
Flux Rate (LMH):	30.00
Diafiltration Volumes:	5.00
Operating Temperature (C):	8.00
Final Conductivity (µS):	0.60
Final pH:	8.80
Yield (%):	95

Process Description

Process Description: The purpose of this step is to change the pH of the HS product pool from pH 5.6 to 8.5 by titrating with 1 M Tris-Bis-Tris Propane, pH 9.5. Once the pH is adjusted to 8.8 the HS pool is diafiltered to reduce the conductivity to 0.6 ms.

Poros PI Column — Step No.: 6

Process Information

Media:	50 um HS
Column Diameter (cm):	20.00
Column Height (cm):	10.00
Loading Linear Velocity (cm/h):	500.00
Eluting Linear Velocity (cm/h):	500.00
Load Protein Concentration (mg/ml):	0.10
Column Binding Capacity (mg/ml):	0.25
Column Yield (%):	60.00

Vendor Information

Chromatographic media is 50 micron Poros PI resin from Perseptive BioSystems

Column and Sample Conditions

Column and elution buffers are at room temperature and the column load is kept at 4 C. The product peak is collected on ice and chilled to less than 4-8 C.

Calculated Parameters

Column Volume (ml):	3,142	Loading Flow Rate (ml/min):	2,618
Total Protein Loaded (mg):	785	Eluting Flow Rate (ml/min):	2,618
Total Load Volume (ml):	7,854	Total Protein Eluted From Column (mg):	471

Column Buffers

PI Equilibration Buffer (A)-T-BTP	g/L
7.5 mM Tris	0.908
7.5 mM Bis-Tris Propane	2.117
5 N HCl to pH 8.5	
Conductivity @ 20 C (u)	0.600

PI Equilibration Buffer (B)-T	g/L
15 mM Tris, pH 8.5	0.182
5 N HCl to pH 8.5	
Conductivity @ 20 C (u)	

PI Elution Buffer (C)-T	g/L
15 mM Tris, pH 8.0	0.182
5 N HCl to pH 8.0	
Conductivity @ 20 C (u)	

PI Elution Buffer (D)-T-BTP	g/L
7.5 mM Tris	0.908
7.5 mM Bis-Tris Propane	2.117
5 N HCl to pH 7.0	
Conductivity @ 20 C	

PI Cleaning Buffer (1)	g/L
1 N NaOH	40.00
1 M NaCl	58.40

PI Cleaning Buffer (2)	g/L
D.I. Water	1000.00

Buffer Requirements Per Cycle

Buffer	Volume (L)
PI Equilibration Buffer (A)	75.40
PI Equilibration Buffer (B)	21.99
PI Elution Buffer (C)	31.42
PI Elution Buffer (D)	21.99
HS Cleaning Buffer (1)	15.71
HS Cleaning Buffer (2)	15.71

Raw Material Requirements Per Cycle

Item	Amount (g or L)
Tris (g)	98.15
Bis-Tris-Propane (g)	206.17
5 N HCl (L)	
NaCl (g)	917.35
NaOH (g)	628.32
D.I. Water (Liters)	142.94

Total Processing Time (min) 54.6

Process

Step	Buffer/Description	C.V.	Total Vol. (L)
1	Equilibrate Column with PI Equilibration Buffer (A)-T-BTP	10.0	31.42
2	Load Column with Diafiltered HS Pool	2.5	7.85
3	Wash Column with PI Equilibration Buffer (A)-T-BTP	4.0	12.57
4	Wash Column with PI Equilibration Buffer (B)-T	7.0	21.99
5	Elute with PI Elution Buffer (C)-T	10.0	31.42
6	Elute with PI Elution Buffer (D)-T-BTP	7.0	21.99
7	Wash Column with PI Cleaning Buffer (1)	5.0	15.71
8	Wash Column with D.I Water (2)	5.0	15.71
9	Equilibrate Column with PI Equilibration Buffer (A)-T-BTP	10	31.42
	Total	45.5	142.94

Process Description: Column is equilibrated with 15 mM Tris-Bis-Tris Propane, pH 8.5. The sample is loaded onto the column. The column is washed with15 mM Tris-Bis-Tris Propane, pH 8.5 for 4 CV. The column is then washed with 15 mM Tris, pH 8.5. The product pool is eluted with 15 mM Tris, pH 8.0. The degraded fragment and other contaminants are eluted with with 15 mM Tris-Bis-Tris Propane, pH 7.0. The column is cleaned with 1 N NaOH and 1 N NaCl, washed with D.I. water and requilibrated with 15 mM Tris-Bis-Tris Propane, pH 8.5.

Note: Store column in 20% by vol. EtOH in D.I. water.